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ESTABLISHMENT OF MICROBIAL POPULATIONS IN STERILE MINE SPOILS AND OVERBURDEN

(First Annual Report, October 1975)

by

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W. A. Kneib
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In Cooperation With

The Intermountain Forest and Range Experiment Station
and the
SEAM Program

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TABLE OF CONTENTS

	Page
INTRODUCTION	1
EXPERIMENTAL DESIGN	4
Field Studies	4
Laboratory Studies	5
MATERIALS AND METHODS	11
Soil Sample Characterization	11
First Algal Bioassay	11
Second Algal Bioassay	15
FIELD STUDY RESULTS - PART I	18
Field Climate	18
Microbial Activity in Blackbird Mine Spoils, Revegetation Plots, and Surrounding Area	20
LABORATORY ALGAL BIOASSAY RESULTS - PART II	25
Bioassay 1	25
Bioassay 2	29
DISCUSSION	35
Field Observations	35
Microbial Bioassays	36
SUMMARY AND CONCLUSIONS	41
ONGOING AND FUTURE WORK	44
REFERENCES CITED	45

TABLE OF CONTENTS (Continued)

Page

APPENDIX A

TENTATIVE ALGAL BIOASSAY TECHNIQUE FOR OPTIMIZING THE USE OF MINE SPOIL AMENDMENTS FOR REVEGETATION 52

APPENDIX B

METHODS FOR CHARACTERIZING SOIL MICROBIAL COMMUNITIES 61

APPENDIX C

DATA AND STATISTICAL ANALYSIS OF DATA FROM BIOASSAYS 1 AND 2 76

LIST OF FIGURES

Figure		Page
1	Blackbird Mine Spoil Revegetation Plot	6
2	Bioassay 1 microbial responses as affected by different treatments	27
3	Bioassay 2 microbial responses as affected by different treatments	31
4	Lucite chamber used in the acetylene-ethylene assay for nitrogenase activity (Nitrogen fixation)	71

LIST OF TABLES

Table		Page
1	Treatments and various levels of treatments employed in the first bioassay (Bioassay 1)	7
2	Treatments and various levels of treatments employed in Bioassay 2.	8
3	Methods for soil sample microbial activity characterization	12
4	pH vs. incremental lime addition to Blackbird minesite soils with time	16
5	Surface soil temperatures at the Blackbird mine . . .	19
6	Microbial activity in samples of soils and spoil material of the Blackbird mine	21
7	The effect of liming on some properties of Blackbird overburden (spoil) and subsoil (topsoil dressing material)	22
8	Data from Bioassay 1 including means, standard deviations, and coefficients of variations	77
9	Data from Bioassay 2 including means, standard deviations, and coefficients of variation	84

ABSTRACT

Field and laboratory analyses of overburden from the Blackbird Mine near Salmon, Idaho showed that microbial communities were limited in biomass and in diversity of microorganisms. Methods of analysis were developed to determine the factors limiting the microbial productivity and possible ecological niches; bioassay methods were developed to assess the means of changing conditions so that stable microbial communities could be established.

Results of field and laboratory studies showed that the limiting factors for soil algal growth were in order of importance: pH, rate of water application, and phosphorus. Other nutrients and heavy metal immobilization by pH adjustment were altered sufficiently by the above three parameters to allow considerable microbial productivity. A simple and convenient short-term bioassay technique was developed for use on soil systems. Methods for characterizing microbial communities in mine spoils are presented in this report.

INTRODUCTION

Modern civilization and the cultural standard it demands depends entirely upon man's ability to extract raw materials and energy from the earth. Only recently has mankind become aware of the need to conserve resources and protect the environment that supports his very existence on earth. The mining process is vital, but by it's very nature it creates a disturbance in natural systems that may sometines require years or centuries to correct by natural processes. The lack of stabilization of mine spoils and overburden may result in air and/or water pollution, thus further degrading the environment beyond terrestrial disturbances and the often unsightly appearance. Replacing vegetation is the most acceptable and the most preferred method for stabilizing mine spoils (Peterson and Monk, 1967). As with any ecosystem, one or more factors prevent the development of stable communities either because their omission limits populations or because their presence inhibits growth. In mine spoils revegetation is often inhibited by the lack of water first, acids (low pH) next, and finally other factors. The bioassays described below were not intended to evaluate water requirements or pH control. Rather, it was intended that these factors could be made saturating or optimum and then other important growth controlling parameters could be evaluated.

Toxicity (mainly due to heavy metals), nutrient imbalance, and lack of a stable microflora often make the revegetation of mine spoils a difficult and lengthy process. The importance of a stable microflora to soil fertility is widely recognized (Alexander, 1971). Microorganisms aid the fertility of a soil by solubilizing nutrients such as phosphorus and trace metals (Zajic, 1969), by fixing and cycling nitrogen (Campbell and Lees, 1967; Bergersen, 1973; Sorensen and Porcella, 1975), decomposing organic matter, and forming humus (Pauli, 1967). The establishment of such a vital component to soil fertility is therefore, a necessary consideration if the revegetation process is to proceed expeditiously.

Microorganisms which include algae, bacteria, and fungi occupy virtually every environment on the earth's surface, including very harsh and barren climates (Alexander, 1971). Thus microorganisms are the likely candidates to begin stabilization of the harsh environment posed by mine spoils. However, where time is an important factor, alteration of the physical/chemical environment by engineering practices (pH adjustment, nutrient addition, etc.) would open a myriad of micro-ecological niches easily occupied by microorganisms that would contribute to the soil fertility. This report presents the progress and findings of the first phase of a study intended to elucidate techniques and requirements for encouraging the establishment of microbial populations in mine spoils and overburden.

In addition, the fundamental inorganic nutrient (C, N, P, K) requirements of algae and higher plants are similar. Thus the microbial bioassay can be used to suggest nutrient additions and other modifications of the mine spoil which will allow revegetation with higher plants. The ease and rapidity of performing microbial bioassays will eliminate much of the screening necessary in higher plant revegetation studies.

The success of revegetation of mine spoils and overburden is inseparably connected with the fertility and toxicity of the material to be revegetated. A rapid, practical method for assessing toxicity and nutrient requirements in mine spoils and related materials would be of considerable value for determining microbial requirements and higher plant growth requirements. Algal bioassays have become a standard tool in aquatic biology (EPA, 1971a; EPA, 1973), yielding valuable information about toxicities and nutrient limitations to productivity in aquatic systems. The application of the basic principles of the algal bioassay with appropriate modification for soil systems has allowed the development of a soil algal bioassay.

EXPERIMENTAL DESIGN

Field Studies

Field and laboratory work in this first phase of the study has concentrated on the Blackbird minesite near Salmon, Idaho. Two approaches to the problem were undertaken in the field. The first involved the sampling and characterization of the microbial activity of the revegetation experimentation currently being conducted by the Forest Service, Intermountain Forest and Range Experiment Station. The second was the field application of laboratory findings (by chemical analysis and bioassays) of important factors in microbial establishment and the effect on higher plants.

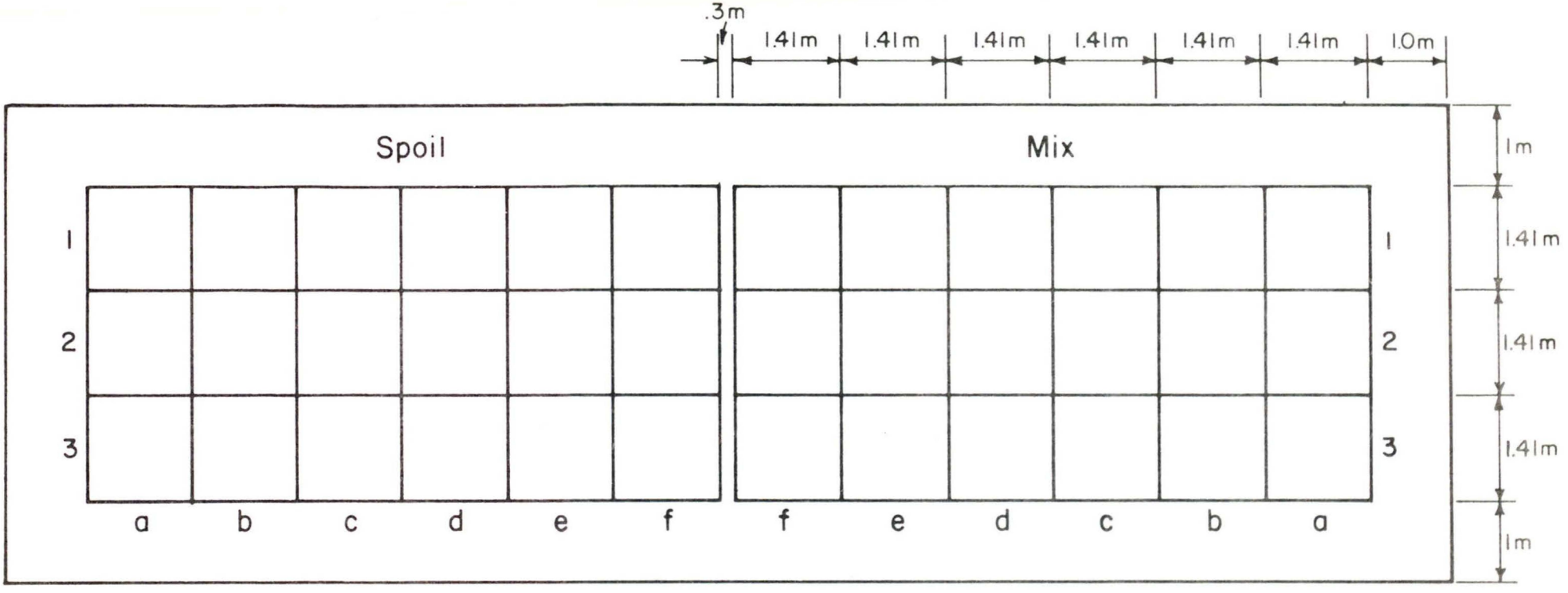
Sampling and analysis of existing revegetation experiments at the Blackbird minesite includes the characterization of microbial activity in the experimental plots used, the evaluation of the effects of irrigation, soil dressing (topsoil covering), fertilizer application (N, P, K), mulch treatment, and the suitability for growth of native plant species and/or introduced pasture grasses. "Off site" revegetation trials using limed and fertilized subsoil (top soil) and mulched-fertilized spoil material were also sampled. A large, ten acre, revegetation project has been undertaken by the Forest Service during the period of this study, and the microbial establishment and population dynamics of this area will be monitored during subsequent study phases.

Testing of applicability of laboratory findings to the field environment has been started with the establishment of an experimental plot on the minesite. This plot was established to evaluate the effects of irrigation, soil dressing (topsoil mixed with spoil), fertilizer application, algal inoculation, and the suitability for growth of native plants. The layout of the plot and the various treatments is shown in Figure 1.

Laboratory Studies

Two sets of soil algae bioassays have been performed on Blackbird mine overburden and a proposed topsoil dressing for that mine. Procedures followed in these bioassays are presented in Appendix A. Table 1 is a schematic diagram of the soil amendment parameters and the level of application examined in the first bioassay. A fairly broad spectrum of amendments and treatments were examined in the first bioassay in order to select those parameters that were most successful in promoting algal growth. The results of the first bioassay were then supplemented with lime requirement data, and used to construct a second bioassay (Table 2) to further evaluate "most promising" amendments and treatments and continue development of the bioassay procedure.

The soil system acts as a matrix in which plants extract nutrients to supply their needs during growth. To the extent that the matrix is unable to supply growth needs or is toxic to growth, amendments will be required. Plants require light, appropriate temperatures, water, carbon, proper pH, nitrogen, phosphorus, and trace elements, Light and temperature are



Row	Treatment	Column	Treatment
1	Algae	a	Control
2	Native Plants & Algae	b	Nitrogen
3	Native Plants	c	Nitrogen & 50 ppm Na_2HPO_4
		d	50 ppm Na_2HPO_4
		e	100 ppm Na_2HPO_4
		f	Nitrogen & 100 ppm Na_2HPO_4

Figure 1. Blackbird Mine Spoil Revegetation Plot (total area is 119.7 m^2 or 1289 sq. ft.).

Table 1. Treatments and various levels of treatments employed in the first bioassay (Bioassay 1).

Amendments	Desert	Subsoil	Spoil *	Mix	Level of Treatment
Control	--	--	--	--	--
L is Lime $Ca(OH)_2$	--	Subsoil L 15 mg $Ca(OH)_2$	Spoil L 95-100 mg $Ca(OH)_2$	Mix L 50 mg $Ca(OH)_2$	$7 < pH < 8$
L N, N is Nitrogen NH_4NO_3	--	Subsoil L N 179 mg NH_4NO_3 15 mg $Ca(OH)_2$	Spoil L N 173 mg NH_4NO_3 95-100 mg $Ca(OH)_2$	Mix L N 176 mg NH_4NO_3 50 mg $Ca(OH)_2$	0.13% total N (w/w)
L TSP, TSP is Phosphorus (Treble super phosphate)	--	Subsoil L TSP 4.4 mg TSP 15 mg $Ca(OH)_2$	Spoil L TSP 95-100 mg $Ca(OH)_2$	Mix L TSP 1.9 TSP 50 mg $Ca(OH)_2$	The subsoil and mix were increased in phosphorus content to 290 and 659 ppm total phosphorus respectively
L Yeast, Yeast is Yeast Extract	--	Subsoil L Yeast 125 mg yeast ext. 15 mg $Ca(OH)_2$	Spoil L Yeast 125 mg yeast ext. 95-100 mg $Ca(OH)_2$	Mix L Yeast 125 mg yeast ext. 50 mg $Ca(OH)_2$	0.25% (w/w)
L Sawdust	--	Subsoil L Sawdust 2.0 g sawdust 15 mg $Ca(OH)_2$	Spoil L Sawdust 2.0 g sawdust 95-100 mg $Ca(OH)_2$	Mix L Sawdust 2.0 g sawdust 50 mg $Ca(OH)_2$	4% (w/w)
L HM, HM is Heavy Metals	--	Subsoil L HM 15 mg $Ca(OH)_2$	Spoil L HM 95-100 mg $Ca(OH)_2$	Mix L HM 50 mg $Ca(OH)_2$	2 ml of trace metal fraction of PAAP solution per liter - this resulting solution is added as water in first wetting ≈ 12.5 ml
L TSP N HM Phosphorus, Heavy Metal, Nitrogen	--	Subsoil L TSP N HM **	Spoil L TSP N HM **	Mix L TSP N HM **	**
L TSP HM	--	Subsoil L TSP HM **	Spoil L TSP HM **	Mix L TSP HM **	**

Note: Number of replicates = 3, sample size = 50 g.

*Already contains 23 ppm HCO_3^- extractable PO_4 and 1130 ppm total phosphorus

**Levels of each constituent are the same as those above

Table 2. Treatments and various levels of treatments employed in Bioassay 2.

Amendments	Subsoil	Spoil	Level of Treatment
Control	---	---	---
L is Lime $Ca(OH)_2$	Subsoil L 15 mg $Ca(OH)_2$	Spoil L 350 mg $Ca(OH)_2$	$7 < pH < 8$
L C is Lime $CaCO_3$	Subsoil L C 200 mg $CaCO_3$	Spoil L C 8000 mg $CaCO_3$	$7<pH<8$, Ca^{++} in lime & $CaCO_3$=40/74 and 40/100
L RGP, RGP is Phosphorus Na_2HPO_4 (25 ppm)	Subsoil L RGP 1 10.8 mg Na_2HPO_4 15 mg $Ca(OH)_2$	Spoil L RGP 1 10.8 mg Na_2HPO_4 350 mg Ca $(OH)_2$	Increased to 298 ppm total phosphorus content in subsoil
L RGP Phosphorus Na_2HPO_4 (50 ppm)	Subsoil L RGP 2 21.6 mg Na_2HPO_4 15 mg $Ca(OH)_2$		Increased to 323 ppm total phosphorus content
L RGP HM Phosphorus Na_2HPO_4 (25 ppm) Heavy Metals	Subsoil L RGP1 HM 10.8 mg Na_2HPO_4 → 15 mg $Ca(OH)_2$	Spoil L RGP1 HM 350 mg $Ca(OH)_2$ 10.8 mg Na_2HPO_4	2 ml of trace metal fraction of PAAP solution per liter - this resulting solution is added as water in first wetting
L RGP HM Phosphorus Na_2HPO_4 (50 ppm) Heavy Metals	Subsoil L RGP2 HM **		**
L HM Heavy Metals	Subsoil L HM **	Spoil L HM 350 mg $Ca(OH)_2$	**
L TSP 1 Phosphorus (4.455 ppm)	Subsoil L TSP 1 1.1 mg TSP		***
L TSP 2 Phosphorus (8.910 ppm)	Subsoil L TSP 2 2.2 mg TSP		***
L TSP 3 Phosphorus (13.365 ppm)	Subsoil L TSP 3 3.3 mg TSP		***
L TSP 4 Phosphorus (17.820 ppm)	Subsoil L TSP 4 4.4 mg TSP		***
L TSP 5 Phosphorus (22.275 ppm)	Subsoil L TSP 5 5.5 mg TSP		***
L TSP 6 Phosphorus (26.73 ppm)	Subsoil L TSP 6 6.6 mg TSP		***

ote: Number of replicates = 4 Sample size = 50 g

*Already contains 23 ppm HCO_3^- extractable PO_4 and 1130 ppm total phosphorus.

**Level of constituent explained above.

***Levels are multiples of this 4.455 ppm addition; based on phosphorus content in Bioassay 1.

environmental variables unassociated with the soil system except that the plant must be exposed to the light (be outside of the soil matrix). Also water and carbon (as CO_2) must be supplied outside the soil matrix and are generally derived from atmospheric sources although water may be in such short supply that watering is required. The following sections relate to the remaining plant growth requirements, all of which are derived from the soil matrix and can be classified as pH adjustment and nutrient requirements.

Lime or lime products are commonly used in revegetation of acidic mine spoils to adjust the pH to a level better tolerated by common higher plants much the same as they are in agriculture (Buckman and Brady, 1969). Also raising the pH to the neutral pH range precipitates toxic heavy metals as their insoluble hydroxides, thereby greatly improving the suitability of normally acid mine spoils for microbial and higher plant growth. The literature has shown and early experimental observations in this research confirmed that the lime requirement even on a short term basis exceeded that of simple neutralization of existing acidity. Experiments were then designed to empirically determine a "minimum" lime requirement for bioassay work and a total or potential lime requirement for field revegetation work. There is a surprising scarcity of information pertaining to methods for determining lime requirements in mineral mine spoils. An existing peroxide oxidation technique (EPA, 1971b) was tried without success in obtaining

consistent results that were acceptable, i.e., greater than or equal to the empirical or natural oxidation data. This led to the initiation of work to develop a technique for determination of potential lime requirement. Bioassay results and the chemical analysis technique were used to establish lime additions appropriate for performing the bioassay while the lime addition research was continued. The results of this work will be presented in a separate report (Anderson et al., Determination of lime requirement for revegetation of acid mineral mine spoils. In progress).

Fertilizer additions (nutrients) were made to determine imbalance of nutrients and to estimate necessary amendments for field trials.

MATERIALS AND METHODS

Soil Sample Characterization

Methods selected for characterization of microbial activity in soil samples are generally techniques which measure a relatively broad spectrum of microbial activities or growth. The techniques employed and their microbial indications are summarized in Table 3. A brief outline of procedures for use of these methods is given in Appendix B.

First Algal Bioassay

Fifty-gram aliquots of soils screened to pass a 12 mesh Tyler standard screen were amended as shown in Tables 1 and 2 and placed in 100 x 15 mm standard plastic Petri dishes for algal bioassays.

The first bioassay was conducted at room temperature (25 ± 5 C) and 2150 ± 10% lux cool white flourescent illumination. Moisture content of the soils was maintained near 20% by bringing the total weight of the petri dish bottom and soil to a known value (70 g) with deionized distilled water every other day.

The bioassay (Table 1) was conducted for 5 weeks (6 March to 10 April 1975). Soil systems evaluated in the first bioassay included a Great Basin desert soil control, a subsoil (topsoil) intended for surface dressing of spoil material, typical surface material from the Blackbird open pit overburden dump (spoil), and a 1:1 mixture of the spoil and top

Table 3. Methods for soil sample microbial activity characterization.

Technique	Microbial activity measured	Types of organisms involved
Chlorophyll a content	Soil algae activity, i.e., photosynthetic activity (including changes with time)	Chlorophyta (green algae) Cyanophyta (blue-green algae)
Fluorescent microscopy	Quantitative algae counts. Qualitative population analysis of fungi	Algae, fungi, (can be expanded to include bacteria)
Dehydrogenase Activity	Total endogenous microbial respiration	Bacteria, fungi, algae, etc.
Acetylene Reduction	Nitrogen fixation (nitrogenase).	Blue-green algae, bacteria (Azotobacter, Clostridium, etc.), fungi
Nitrogen Content	Nitrogen accumulation or loss (Immobilization mineralization, volitalization)	Algae, bacteria, fungi
Extractable PO_4	Phosphorus availability phosphorus solubilization (Chemical-physical factors also involved)	All microorganisms (This measurement reflects mainly nutrient availability and to some extent microbial activity)

soil (mix). A desert control soil collected from southern Curlew Valley, Utah was included in the first bioassay as a bioassay -physical-condition control. The investigators have had experience dealing with the natural microbial communities of this soil (Sorensen and Porcella, 1975) and felt that any inhibition or other anomaly due to the conditions imposed in the bioassay could be recognized in this soil system.

Measurements of pH throughout the study were made with a pH meter calibrated with known pH buffer solutions. Methods of pH measurement generally conform with USDA Forest Service Research note, NE-98 and NE-154 (Czapowsky and Sowa, 1973). Adjustment of pH for the acidic soil systems (spoil, top soil, and mix) for the first bioassay was made with commercial grade hydrated lime ($Ca(OH)_2$). The lime requirement was estimated empirically by incremental addition of small weights of lime to a 50% weight/volume mixture of the soil material with deionized distilled water until a stable (30 minute) pH of 7-8 was obtained. This method of lime requirement estimation was later determined to be inadequate (see results) due to its inability to compensate for potential acid production from continuing sulfide mineral oxidation. The desert control received no lime, the top soil received 15 mg lime/50 g soil, the spoil received 95-100 mg lime/50 g soil, and the mix received 50 mg lime/50 g soil.

Nitrogen and phosphorus were added to the soils in the first bioassay (Table 1) at levels sufficient to increase the nutrient status to a level

similar to that of the desert control, i.e., 0.13% total nitrogen and 20 ppm bicarbonate extractable orthophosphate. Reagent grade NH_4NO_3 was added as the nitrogen source and a commercial phosphate fertilizer (minimum 45% water soluble P_2O_5) was added for phosphorus supplementation. Soil nitrogen content was monitored throughout the study as total micro-Dumas nitrogen (Coleman model 29 total nitrogen analyzer. 1968). Bicarbonate extractable phosphorus was measured using standard techniques for extraction (Bureau of Reclamation, 1967) and the Murphy-Riley ascorbic acid method (APHA, 1971) for analysis of phosphate in the extract.

Other amendments were added to the bioassays to assess the requirements and/or stimulatory role of complex organic and vitamin addition (yeast extract), mulch and cellulose carbon source (sawdust), and trace metal nutrients (heavy metals). The heavy metals were added in solution in the initial watering to the following approximate final concentrations in the soil materials: Boron 0.04 μg B/g, Manganese 0.15 μg Mn/g, Zinc 0.02 μg Zn/g, Cobalt 5×10^{-4} μg Co/g, Copper 4×10^{-6} μg/g, Molybedenum 0.004 μg Mb/g. With the heavy metal solution was added approximately 0.3 μg Na_2 EDTA·$2H_2O$/g as a chelating agent (EPA, 1971a).

Sawdust was added (2 g/50 g) to simulate a woody mulch and to see if a complex carbon source such as wood shavings would be stimulatory to microbial activity. Results were not favorable in the first bioassay (see results below), and so this treatment was

eliminated in the second bioassay. Another complex form of nutrients, yeast extract, was added as a control for all other nutrients at about 0.25% (weight/weight) and was found to be stimulatory (see results below) to microbial growth, but was not significantly better than phosphorus addition, and was therefore eliminated from the second bioassay. Also, it is not likely that this type of amendment could be made economically in large scale revegetation work.

Second Algal Bioassay

Table 2 describes the set-up of the second bioassay as well as the various amendments tested. The second bioassay was conducted for 6 weeks (9 May 1975 to 24 June 1975). Soil systems evaluated in the second bioassay included the top soil and spoil used in the first bioassay. A lower and better controlled temperature of 24 ± 2 C was used in the second bioassay. Illumination and moisture were maintained the same as in the first bioassay.

Lime addition was made on the basis of an emperical "minimum" lime requirement which was determined by periodically adding hydrated lime to soil material over a 12 day period in order to maintain the pH near neutrality. Table 4 presents the results of this experiment. On the basis of these results and the observations from the first bioassay, 15 mg and 350 mg hydrated lime was added to each 50 g topsoil and spoil bioassay plate, respectively. A very large amount of calcium

Table 4. pH vs. incremental lime addition to Blackbird minesite soils with time.

Time Elapsed		Subsoil		Spoil (1)		Spoil (2)		Spoil (3)		Mix* (1)		Mix (2)		
Days	Minutes	Increment of lime added (mg)	pH	Increment of lime added (mg)	pH	Increment of lime added (mg)	pH	Increment of lime added (mg)	pH	Increment of lime added (mg)	pH	Increment of lime added (mg)	pH	Comments
--	--	0	5.5	0	3.6	0	3.6	0	3.6	0	4.0	0	4.0	Sample size is 50
0	0	15	7.3	100	6.6	120	7.0	150	8.0	50	5.8	75	7.5	
0	10		7.5		6.6		6.4		7.8		5.8		7.1	
0	20		7.6		6.1		6.4		7.6		6.0		7.0	
0	40		7.5	60	7.6	50	7.7		6.9	35	7.3	5	6.9	
0	60		7.5		7.4		7.5		6.8		7.3		6.9	
0	90		7.5	30	8.3	40	8.4	20	7.4	10	7.4	20	7.7	During this elaps time, samples we
~ 2	0		6.9		6.2		6.4		6.0		6.3		6.4	flooded and stagn
2	40	10	8.1	40	7.7	40	7.8	70	8.2	35	8.0	40	8.3	
2	85		7.9		7.3		7.5		7.8		7.7		7.9	
2	130	15	8.3	40	8.3	50	8.6	50	8.7	30	8.6	30	8.8	During this elaps time the samples
~ 5	0		7.5		7.0		7.1		7.2		7.3		7.4	dried out.
5	30	20	8.9	50	8.6	50	8.8		7.2	40	8.9		7.4	
~12	0		7.9		7.6		7.6		7.4		7.7		7.5	
Total lime added (mg)		60		320		350		290		200		170		

*1:1 mixture of spoil and subsoil.

carbonate ($CaCO_3$) was added to the soils of one set of assay plates (200 mg/50 g topsoil, and 8 g/50 g spoil) as a total required lime addition test.

Nitrogen addition was eliminated from the second bioassay because of seemingly non-stimulatory results in the first bioassay.

Phosphorus was added in the second bioassay as reagent grade dibasic sodium phosphate (Na_2HPO_4). In addition, commercial phosphate fertilizer was added in amounts of from 1.1 to 6.6 mg/50 g soil to observe the effects of increasing or decreasing the level of phosphorus used in the first bioassay (4.4 mg/50 g soil). In both the first and second bioassays phosphorus was added as a supplement to existing phosphorus levels to achieve the concentrations listed in Tables 1 and 2.

Heavy metal trace nutrients and EDTA were added as in the first bioassay to the same levels used in the first bioassay. No other special amendments were used in the second bioassay.

FIELD STUDY RESULTS - PART I

Field Climate

Temperature and water availability have profound effects on microbial activity in any environment. During the summer of 1974 a continuously recording soil temperature monitor and a fence post cumulative precipitation gage were installed. Daily surface soil temperatures at the minesite are shown in Table 5. Extreme temperature variations of 30 C or more each day indicate a harsh environment that may limit the ability of some microorganisms to function effectively Cumulative precipitation at the minesite was recorded as 4.32 cm (1.7 inches) from 23 July 1974 to 27 August 1974 and 0.76 cm (0.3 inches) from 27 August 1974 to 9 October 1974. Rainfall during this period was somewhat below the normal precipitation expected for average summer months but probably represent the order of magnitude of precipitation that can be expected during summer months. Therefore, the surface soils, in which the majority of microbial activity can be expected to occur, present a stressed environment of infrequent moisture addition and very high to very low diurnal temperature variation. Microorganisms exhibit an almost unbelievable ability to adapt to environmental extremes, but irrigating revegetation areas probably hastens microbial establishment and hence increased fertility of the soil matrix. This would serve to both make water available to biological activity and buffer high temperatures through evaporative cooling.

Table 5. Surface soil temperatures at the Blackbird mine (record is incomplete due to instrument malfunction).

Date Mo.-Day-Year	Maximum °C	Minimum °C
7-24-74	41.5	10.5
7-25-74	42.5	11.5
7-26-74	41.5	11.5
8-29-74	33.5	--
8-30-74	33.5	7.5
9-1-74	32.5	6.5
9-2-74	30.2	6.0
9-3-74	32.0	1.0
9-4-74	32.5	6.5
9-5-74	32.0	8.0
9-6-74	30.5	6.0
9-7-74	31.0	5.5
9-8-74	32.0	6.5
9-9-74	30.5	11.5
9-10-74	30.5	7.0

Microbial Activity in Blackbird Mine Spoils, Revegetation Plots, and Surrounding Area

Table 6 lists the results of microbial activity characterization analysis done on field soil samples collected at the Blackbird minesite. The unamended overburden material (spoil) from the open pit mine appears to be very low in diverse microbial activity. Algae and fungi were not observed, and dehydrogenase activity indicates that total endogenous microbial respiration is very low. The low pH and the production of acid from this material indicates that chemotrophic bacteria such as Thiobacillus ferrooxidans, T. thiooxidans, Ferrobacillus ferrooxidans, etc. are probably present in the spoil material. These organisms are capable of oxidatively metabolizing sulfide minerals such as chalcopyrite ($CuFeS_2$), pyrite (FeS_2), arsenopyrite (FeAsS), and pyrrhotite (Fe_nS_{n+1}) with the production of sulfuric acid. All of the above sulfide minerals and others have been reported in the Blackbird mine district (Anderson, 1947). It has been demonstrated that under aerobic conditions these bacteria are capable of accelerating the oxidation of pyrite with the production of acid ten fold over the purely chemical oxidation process (FWPCA, 1970). This phenomenon contributes greatly to the need to apply sufficient lime or lime products to acid mine spoils and overburden to neutralize the acid continually being produced. This may be thought of as a total lime requirement. The effect of liming on the soluble heavy metals of this overburden material is shown in Table 7. The precipitation of the soluble heavy

Table 6. Microbial activity in samples of soils and spoil material of the Blackbird mine area. Note: Acetylene reduction (nitrogenase) activity was not detected.

Sample	Description	Collection Date	Chlorophyll a μg/g	Microscopy Algae #/g	Microscopy Fungi[a] +/−	Dehydrogenase activity[b] μg formazan/g	Total Nitrogen(%)	Extractable[c] PO_4 (ppm)	pH
Spoil	Unamended	31 July 74							4.3-4.5
		27 Aug 74	ND	<800	--	ND	0.003		
		10 Oct 74	ND	<800	--	0.2	0.009	23	
Mulched Spoil	(Forest Service	27 Aug 74	INT	<1000	–	1.0	0.050		
	off-site experiment)	10 Oct 74		<110	--	3.4	0.046		
Top Soil	(Forest Service	27 Aug 74	0.4	4600	+	4.5	0.045		
	off-site experiment)	10 Oct 74	0.37	220	+	6.8	0.047		
Forest Floor		27 Aug 74				17.0			
		10 Oct 74	INT	1500	+	1.0	1.250		
BBTS2 NP-NM-NF*	Forest Service Plot	23 July 75				2.1	0.011		
BBTS3 NP-M-F	Forest Service Plot	23 July 75				8.0	0.066		
BBTS4 P-NM-F	Forest Service Plot	23 July 75				8.3	0.038		
BBTS5 P-M-NF	Forest Service Plot	23 July 75				11.6	0.031		
BBTS6 P-NM-NF	Forest Service Plot	23 July 75				<0.1	0.004		
BBTS7 N-M-NF	Forest Service Plot	23 July 75				11.2	0.023		
BBTS10 P-M-NF	Forest Service Plot	23 July 75				4.4	0.016		
BBTS11 N-M-F	Forest Service Plot	23 July 75				14.8	0.059		
BBTS12 NP-M-NF	Forest Service Plot	23 July 75				13.8	0.018		
BBTS13 P-M-F	Forest Service Plot	23 July 75				3.8	0.090		
BBTS17 N-NM-F	Forest Service Plot	23 July 75				4.4	0.030		
BBTS20 NP-NM-F	Forest Service Plot	23 July 75				13.1	0.060		
BBTS21 N-NM-NF	Forest Service Plot	23 July 75				<0.1	0.006		
BBTS30 NP-M-NF	Forest Service Plot	23 July 75				1.9	0.018		
Upper Bench	(Forest Service Large	9 Oct 75	<0.02			2.1	0.020		7.4
Lower Bench	Revegetation Area)	9 Oct 75	0.08			<0.3	0.013		7.5

[a]Presence or absence of observation of fungal mycelia.

[b]Formazan refers to 2,3,5 triphenyltetrazolium formazan, the reduced product of the dehydrogenase test (See Appendix).

[c]Bicarbonate extractable orthophosphate.

*BB = Blackbird mine site; TS2 = Topsoil dressed - A Forest Service Plot Number (2,3, . . 36). NP = Native, pasture grasses mixed, N = Native plants, P = Introduced pasture grasses, M = Mulched, NM = No mulch, F = Fertilized, NF = Not fertilized, INT = interference.

Table 7. The effect of liming on some properties of Blackbird overburden (spoil) and subsoil (topsoil dressing material).

Material	Lime Added	% Water*	EC_e mmhos/cm at 25°C	Cation Exchange Capacity, meg/100g	$HClO_4$ % P	NO_3-N ppm
Spoil	0	36	1.3	12.0	0.05	12.5
Spoil	700 mg/100 g	37	2.9	13.2	0.04	14.0
Subsoil	0	29	0.3	5.2	0.02	1.2
Subsoil	30 mg/100 g	30	0.4	No date	0.02	0.8

		Water-soluble in saturated extract (mg/l)**										
Material	Lime Added	Cu	Fe	Mn	Zn	B	Mg	Ca	Na	K	Pb	Cl
Spoil	0	14.4	28.0	1.21	1.58	<.1	22.6	46.8	50.5	63.4	<.1	44.3
Spoil	700 mg/100 g	<.05	<.1	<.1	.10	<.1	5.9	700.	22.7	26.9	<.1	61.2
Subsoil	0	<.05	<.1	<.1	.21	<.1	5.3	21.0	17.3	4.5	<.1	48.9
Subsoil	30 mg/100 g	<.05	<.1	<.1	.43	.2	9.8	49.3	17.3	3.7	<.1	34.3

*At saturation or field capacity.

**Obtained from a water saturated paste of 100-200 g sample. USDA Handbook 60.

metals would greatly increase the suitability of this material for microbial growth and higher plant vegetation.

Samples from a spoil area that had been mulched (Table 6, Mulched Spoil), limed, fertilized, and seeded with pasture grasses showed increased microbial respiration activity (dehydrogenase activity). No other indications of significant improvement in microbial diversity or activity were observed.

The "topsoil" samples were collected from an area of the overburden dump that had been covered with soil used by the forest service in its soil dressing experimental plots. The soil had been limed, fertilized, and planted to pasture grass. As can be seen in Table 6, chlorophyll a, microscopy, and dehydrogenase activity indicate that this soil system supports a well diversified and active population of microorganisms.

The forest floor litter and soil were also sampled and analyzed. As was expected, analyses indicated an actively respiring (high dehydrogenase activity) diversified population of microorganisms including algae and fungi. Probably due to the high organic content of the soil and litter, dehydrogenase activity was very high when sampled during August. The colder temperatures or perhaps other environmental factors seem to have caused a decrease in respiratory (dehydrogenase) activity in October.

Partial characterization of samples from fourteen Forest Service experimental plots (Table 6) reveals that dehydrogenase activity is substantial in many of the topsoil dressed experimental plots. Linear regression analysis shows essentially no correlation ($r^2 = 0.16$) between nitrogen content and dehydrogenase activity in these plot soils. However, when nitrogen content is very low (BBTS6 P-NM-NF and BBTS21 N-NM-NF) dehydrogenase activity is also very low (< 0.1 μg formazan/g).

During September 1975 the Forest Service Intermountain Forest and Range Experiment Station, Logan, Utah began a 10 acre (4 ha.) revegetation area on the Blackbird open pit mine dump. Samples were collected from the upper and lower benches of this area (Table 6) after the topsoil dressing had been applied, limed, and fertilized but not seeded. These initial values will be used as baseline data in tracing the development of microbial populations in this soil. Possibly, plant growth and development can be correlated with microbial activity and population dynamics and/or chemical-physical observations during subsequent phases of the study.

LABORATORY ALGAL BIOASSAY RESULTS-
PART II

The results of analyses (pH, total N, N fixation, Chlorophyll a, dehydrogenase, and extractable P) performed on the various soil treatments at the end of both bioassays are tabulated in Appendix C (Tables 8 and 9).

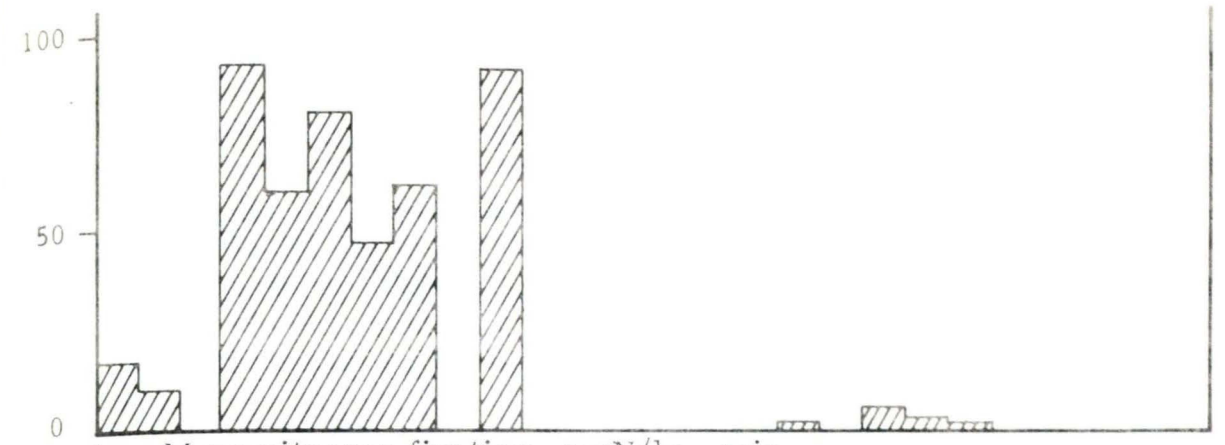
Bioassay 1 (Figure 2)

Inadequate pH control (under liming) in the spoil and spoil-subsoil-mix treatments in the first bioassay resulted in acid conditions and toxicity. Little or no nitrogen fixation or dehydrogenase activity was measured. The growth of an algal species tentatively identified as Chlorella elipsoidea on the acid spoil material resulted in a measurable but very small amount of chlorophyll a being produced in three of the spoil treatments. Since no other algal species grew on the spoil, this organism must have been selected for its tolerance to low pH and soluble heavy metals. Some blue-green algae were able to achieve limited growth on a few mix treatments, but the magnitude of the overall response was insignificant. Good microbial response was observed in several of the subsoil (soil dressing material) tests in the first bioassay. Significant statistical difference in response between treatments in both the first and second bioassays was established at the 95% confidence limits using a t test (Ostle, 1963). (Appendix C, Tables 10, 11, 12, and 13 list values of t for these comparisons.) From Figure 2A it appears that the addition of phosphorus (treatment 4), yeast extract (treatment 6), and

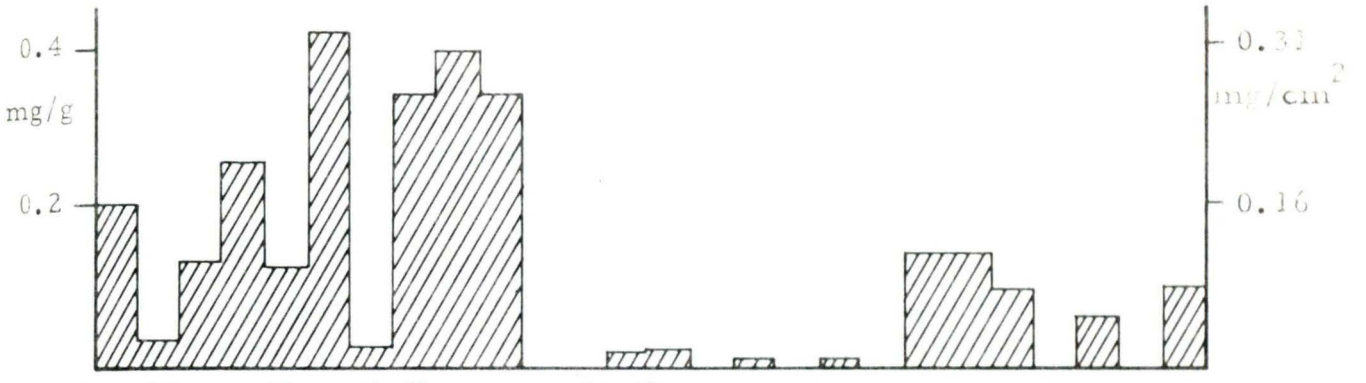
Treatments employed in bioassay 1 and their corresponding number designation as they appear in Figure 2 (facing page).

No.	Treatment
1	Desert Control
2	Subsoil Control
3	" + 15 mg lime, 179 mg NH_4NO_3
4	" + " , 4.4 mg TSP*
5	" + 15 mg lime
6	" + " , 125 mg yeast
7	" + " , 2.5 g sawdust
8	" + " , Heavy metals
9	" + " , 179 mg NH_4NO_3, 4.4 mg TSP Heavy metals
10	" + " , 4.4 mg TSP, Heavy metals
11	Spoil Control
12	Spoil + 100 mg lime, 173 mg NH_4NO_3
13	" + 100 mg lime
14	Spoil + 100 mg lime, 125 mg yeast
15	" + " , 2.5 g sawdust
16	" + " , Heavy metals
17	" + " , 173 mg NH_4NO_3, Heavy metals
18	Mix Control
19	Mix + 50 mg lime , 176 mg $NH_4 NO_3$
20	" + " , 1.9 mg TSP
21	" + 50 mg lime
22	" + 50 mg lime, 125 mg yeast
23	" + " , 2.5 g sawdust
24	" + " , Heavy metals
25	" + " , 176 mg NH_4NO_3, 1.9 mg TSP, Heavy metals
26	" + " , 1.9 mg TSP, Heavy metals

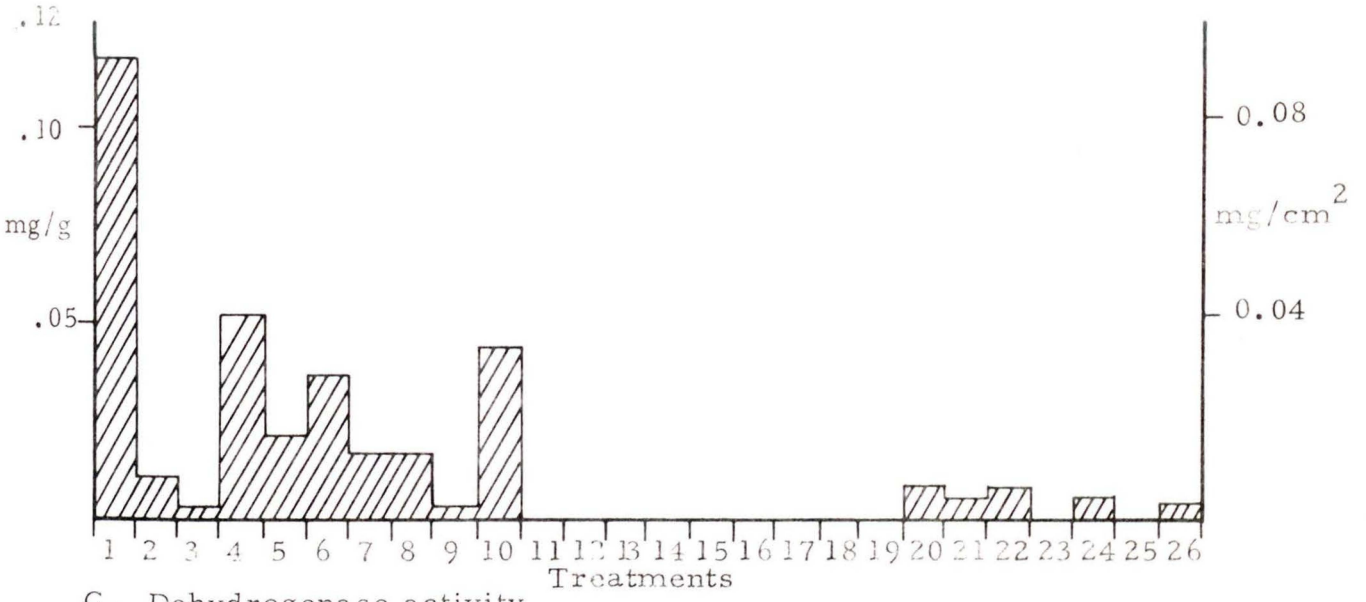
*TSP = Treble Super Phosphate commercial phosphate fertilizer.



A. Mean nitrogen fixation, mgN/l a. min.



B. Mean chlorophyll a concentration



C. Dehydrogenase activity

Figure 2. Bioassay 1 microbial responses as affected by different treatments.

phosphorus plus heavy metals (treatment 10) to limed subsoil results in a high nitrogen fixation capacity. However, the statistical comparison does not show these values to be significantly different from simple lime addition (treatment 5). The addition of sawdust (treatment 7) and the heavy metal solution (treatment 2) to the limed subsoil resulted in a nitrogen fixation capacity significantly below treatments with phosphorus and yeast extract. The addition of ammonium nitrate to limed subsoil (treatments 3 and 9) prevented any measurable nitrogen fixation.

Chlorophyll a production in the limed subsoil (Figure 2B) significantly improved by the addition of phosphorus (treatment 4), yeast extract (treatment 6), nitrogen plus phosphorus plus heavy metals (treatment 9), and phosphorus plus heavy metals (treatment 10). The addition of the heavy metal solution to the limed subsoil (treatment 8) did not result in a significant improvement in chlorophyll a production over simple lime addition (treatment 5) even though the mean chlorophyll a concentration is higher. Treatment of the limed subsoil with ammonium nitrate alone (treatment 3) did not result in significantly improved chlorophyll a production over lime addition alone (treatment 5). Lime addition to the subsoil (treatment 5) did significantly improve chlorophyll a production over the control (treatment 2). Mulching the limed subsoil with sawdust (treatment 7) did not produce a chlorophyll a concentration significantly different from the control (treatment 2).

Dehydrogenase activity was measured on a composite of equal weights from the three replicates in each treatment (see Appendix C) because of time and expense involved in the analytical procedure. Therefore, only a single value representing the mean dehydrogenase activity of each treatment is available and statistical comparison of the values is not possible. Visual inspection of Figure 2C shows that treatments 4 (lime and phosphorus), 6 (lime and yeast extract), and 10 (lime and phosphorus and heavy metals) resulted in higher dehydrogenase activities in the subsoil material. All dehydrogenase values were obviously below that of the actively respiring Curlew Valley desert soil (treatment 1).

Bioassay 2 (Figure 3)

Better temperature control in the second bioassay (24 ± 2 C) over the first bioassay (26 ± 5 C) and better moisture control in the second bioassay resulted in a general improvement in microbiological response over the first bioassay. Nitrogen fixation was significantly higher in the limed and amended spoil material than any of the subsoil treatments with the exception of treatment 17 (8000 mg $CaCO_3$). The addition of this large amount of calcium carbonate gave a nitrogen fixation rate that was not significantly better than the subsoil with phosphorus additions (treatments 4, 5, and 6). The increase in nitrogen fixation rate by the spoil material over the subsoil coincides with the microscopic observation that without exception the algal material growing

Treatments employed in bioassay 2 and their corresponding number designation as they appear in Figure 3 (facing page).

No.	Treatment
1	Subsoil Control
2	" + 15 mg lime
3	" + 200 mg $CaCO_3$
4	" + 15 mg lime, 21.6 mg $Na_2HPO_4 \cdot 7H_2O$
5	" + 15 mg lime, 10.8 mg "
6	" + " , 21.6 mg " , Heavy metals
7	" + " , 10.8 mg $Na_2HPO_4 \cdot 7H_2O$, Heavy metals
8	" + " , Heavy metals
9	" + " , 1.1 mg TSP*
10	" + 15 mg lime, 2.2 mg TSP
11	" + " , 3.3 mg TSP
12	" + " , 4.4 mg TSP
13	" + " , 5.5 mg TSP
14	" + " , 6.6 mg TSP
15	Spoil Control
16	" + 350 mg lime
17	" + 8000 mg lime
18	" + 350mg lime, 10.8 mg $Na_2HPO_4 \cdot 7H_2O$
19	" + " , 10.8 mg $Na_2HPO_4 \cdot 7H_2O$, Heavy metals
20	" + 350 mg lime, Heavy metals

* TSP = Treble Super Phosphate commercial phosphate fertilizer.

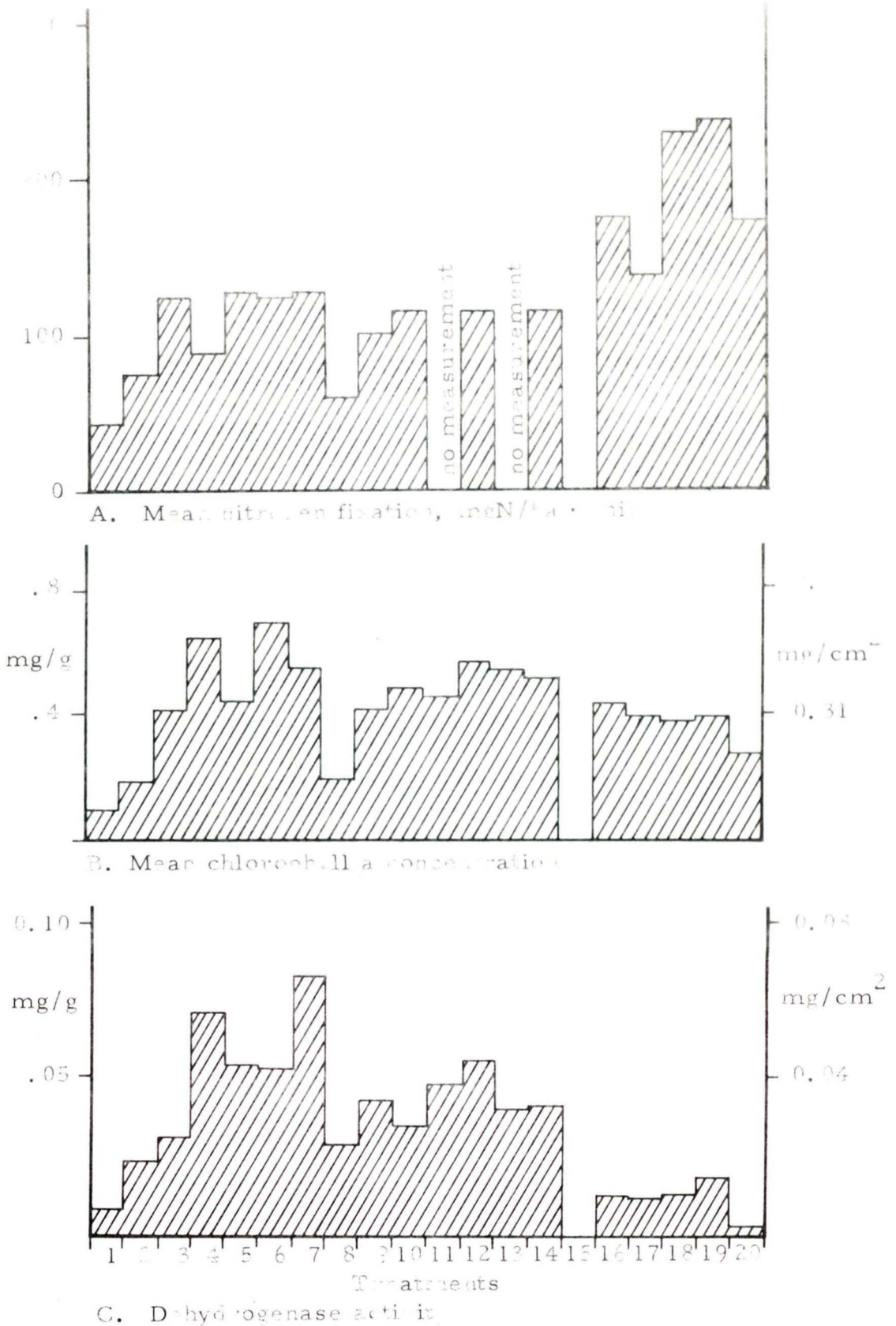


Figure 3. Bioassay 2 microbial responses as affected by different treatments. Note increases in scale in A and B over Figure 2 A and B.

on the spoil was composed of about 80% to 90% Nostoc sp. - a highly heterocystous nitrogen fixing blue-green alga. The algal material growing on the subsoil was of more diversified species, many of which were non-heterocystous blue-green algae (non-nitrogen fixers). The addition of 25 ppm phosphorus with and without heavy metals to the limed spoil accelerated nitrogen fixation (treatments 18 and 19) without significant difference between the treatments. The addition of heavy metals to the limed spoil alone (treatment 20) did not produce significant difference in nitrogen fixation from that of lime addition alone (treatment 16).

Nitrogen fixation in the subsoil material that received amendments (not the control, treatment 1) was not significantly different in any treatment except simple lime addition (treatment 2) and lime and heavy metals addition (treatment 8). These two treatments (2 and 8) were significantly below all others. The addition of a gradient of commercial phosphate fertilizer (treatments 9 to 14) failed to produce a significant difference in nitrogen fixation response.

Chlorophyll a production was generally higher in the subsoil material. Treatment 6 (lime and 50 ppm phosphorus and heavy metals) produced chlorophyll a concentrations significantly higher than most of the treatments but was not significantly better than treatments 4(lime and 50 ppm phosphorus), 7 (lime and 25 ppm phosphorus and heavy metals), 12 (lime and 17.3 ppm commercial phosphorus), 13 (lime and

21.6 ppm commercial phosphorus), and 14 (lime and 25.9 ppm commercial phosphorus). Heavy metal addition to the limed subsoil (treatment 8) was not significantly different in chlorophyll a production from lime addition alone (treatment 2).

The production of chlorophyll a on the spoil material was very uniform with no significant difference between any treatment except the control (treatment 15). This indicates that phosphorus addition does not significantly increase the production of chlorophyll a (algal biomass) on the spoil material. Generally, there is not a significant difference between chlorophyll a production on the spoil and subsoil material. This is especially true if treatment 20 (lime and heavy metals) is eliminated from the spoil treatments. When this is done only treatment 6 (subsoil and lime and 50 ppm phosphorus and heavy metals) and 12 (subsoil and lime and 17.3 ppm commercial phosphorus) are consistently significantly better than the spoil treatments (16, 17, 18, and 19).

Examination of Figure 3C, indicates that microbial respiration (dehydrogenase) in all of the limed subsoil material to which phosphorus was added was about the same irregardless of the level of addition employed. Treatments 4 (lime and 50 ppm phosphorus) and 7 (lime and 25 ppm phosphorus and heavy metals) appear to be higher in activity than the other treatments, but it is difficult to say whether they are truly significantly improved over the other phosphorus addition

treatments. Dehydrogenase activity in the spoil treatments is lower than the subsoil treatments. This indicates that some inhibition of heterotrophic microorganisms may be present in the amended spoil material.

Inspection of photographs taken of the soil surface in each of the various treatments during both bioassay 1 and 2 indicate that phosphorus addition to both the subsoil (topsoil dressing) and spoil materials resulted in more rapid algal growth (35 mm color slides available at UWRL). Increasing the amount of phosphorus added to the subsoil (Bioassay 2) hastened the development of algal growth visible to the unaided eye, i.e., the 50 ppm addition of phosphorus showed faster algal growth than 25 ppm addition of phosphorus. Thus, even though there was not a statistically significant difference between the addition of 25 or 50 ppm phosphorus in nitrogen fixation rate or chlorophyll a concentration at the end of the bioassay, there was an accelerated rate of growth initially by adding more phosphorus.

DISCUSSION

Field Observations

The development of microbial activity in the surface soils at the Blackbird minesite is faced with a relatively harsh climate of extreme temperature variation both seasonally and on a diurnal basis; precipitation during the short summer growing season is irregular and sparse. However, analyses of various treatments presently being evaluated by the Forest Service at the Blackbird minesite shows that microbial communities have been and are developing where chemical-physical conditions have been made favorable. The subsoil material employed by the Forest Service as a topsoil dressing provides a good matrix for microbial development. When the pH of the subsoil is controlled near neutrality by lime addition and chemical fertilizers (N, P, K) are added, good microbial activity seems to develop. Algal, fungal, and bacterial components of the microbial community have been observed in subsoils treated in this way (Table 6).

Limited data have been collected on the microbial response to treatments of the uncovered spoil material. Samples from the treated spoil (limed, fertilized, and mulched with wood fibre, Table 6 "Mulched Spoil") indicate that microbial development is slower in this material. However, laboratory bioassays seem to indicate that good microbial activity can be developed in the spoil material if treatments are sufficient to control pH near neutrality and supply sufficient inorganic and organic nutrients.

Microbial Bioassays

The ability of the subsoil and spoil materials used in the microbial bioassays to support and encourage microbial establishment and activity is primarily dependent on pH adjustment and control away from the natural acidity. Water was not a variable, since it was added in great quantity so that it was not limiting. The first bioassay demonstrated the need for sufficient lime addition to neutralize existing acidity and to consume acid produced by microbial oxidation of sulfide minerals in the material. The majority of microorganisms that will contribute to the fertility of the soil are unable to grow at low pH and are unable to tolerate the high levels of soluble metals in the acid spoil material.

Once pH had been controlled, the addition of phosphorus to the subsoil material seemed to stimulate microbial development as well or better than any other amendment tried. It is interesting to note that the addition of a complex organic, yeast extract, did not significantly stimulate either algal (autotrophic) or heterotrophic (dehydrogenase) activity above that of phosphorus addition. It is possible that the algal component of the bioassay system provides sufficient organic material so that heterotrophic microbial activity is stimulated. One can conclude that components other than lime, water, and phosphorus are unnecessary for stimulating microbial growth. Also, calculations show that nitrogen fixation would add 10.7 lbs of nitrogen per acre (12 kg/ha)

annually assuming a 60 day growing period with 14 hours of daylight and optimum fixation rates (240 mg/ha min). Ideally, the growth of algae on the soil in the field as in the laboratory would provide a self-perpetuating, low management input of organic matter that would be available for heterotrophic metabolism and humus formation.

The amount of nitrogen added in the laboratory bioassays by the nitrogen fixing algae appears comparable to imputs in natural terrestial systems (see review in Sorensen and Porcella, 1975) is considerably relative to agricultural applications. Typically, nitrogen additions in cropland in Idaho are 50-100 lbs/acre and average 26 lbs/acre for all US cropland and total N fertilizer usage (USDA, 1967). The Forest Service used amendments of 78 lbs/acre in 1972 and an additional 100 lbs/acre of nitrogen in 1973 in their revegetation experiments and the algal inputs would be 10-15 percent of these additions. Thus microbial reestablishment in disturbed soil systems apparently would have significant value in adding a nutrient which commonly limits revegetation and is increasingly more expensive.

Field trials are underway (Figure 1) to see if effective growth of algae can be accomplished on the surface of treated mine spoils at the Blackbird minesite. It is possible that an external source of organic matter for heterotrophic metabolism and humus formation will be needed. The evaluation of barnyard manure for this purpose is presently under evaluation by the algal bioassay technique.

The addition of 25 ppm phosphorus to the limed spoil material in the second bioassay did not result in a significantly higher production of chlorophyll a over that of lime addition alone. It is possible that raising the pH of the spoil material solubilizes ferric phosphates raising the available phosphorus content of the limed spoil material to a level at which it is no longer limiting to algal growth (Stumm and Morgan, 1970). The effects of adding phosphorus at levels above 25 ppm to limed spoil material is being evaluated further.

The addition of nitrogen as ammonium nitrate inhibits microbial activity development. The oxidation of the ammonium ion may produce enough acidity to lower the pH of the soil and thus inhibit microbial growth. These pH problems seemed to occur in the first bioassay when ammonium nitrate was added to the soils bioassayed. However, subsequent trials with pH under better control have shown the inhibition to not be the result of low pH. In bioassay 1 it is noteworthy that chlorophyll a was high but that the other parameters were low. Thus, nitrogen fixation conferred a competitive advantage on blue-green algae in systems where no addition of nitrogen occurred but not in these systems where $NH_4\ NO_3$ was added. Further research is needed to elucidate the cause of this inhibition by nitrogen addition.

The low microbial response to the addition of a sawdust mulch was probably due to the complex organic structure of the wood fibre. The cellulose in the wood fibre is difficult for most microorganisms

to attack. In addition, the sawdust itself shaded the soil surface and made algal growth difficult; hence low nitrogen fixation capacity and low chlorophyll a content.

The very low final concentrations of heavy metals added to the bioassays were probably not significant in stimulating microbial growth in the bioassays. However, the addition of the chelator ethylenediaminetetraacetic acid (EDTA)may have made some trace metal nutrients more available for microbial use and therefore account for what appears to be a slightly better performance (higher mean value) in some of the treatments to which the heavy metal mix was added. EDTA is not a good chelating agent in calcareous soils and soils to which large amounts of lime have been added (Wallace, 1956). The use of other metal chelates of proven value in calcareous soils is presently under evaluation using the micriobial bioassay technique.

In summary there are distinct advantages and disadvantages to microbial bioassays of soil materials whether natural soils, subsoils, or spoils. Advantages include short time period for study, small space requirements, replication for adequate statistical analysis, analysis of factors related to microbial effects on soil systems (acid production, heavy metal mobilization, anaerobiosis, denitrification, and other processes), application to reestablishment of microbial communities in soil systems and to related questions of soil binding, soil permeability, and erosion relationships. Disadvatages are that algae are not equivalent to plants in terms of nutrient accumulation or composition, nitrogen fixation by plants (legumes

and other symbiotic plant nitrogen fixers) probably does not relate closely to quantitative fixation inputs by the blue-green algae, the microbial communities of the shallow soil bioassays are probably more active than in deeper soil profiles. However, the bioassay does indicate that nitrogen fixation by plant-bacterial systems is feasible. In our opinion the soil bioassay adds a great amount of information of microbial dynamics and allows a significant screening of nutrients which might be valuable in designing a plant-fertilization experiment. Assuredly, it is necessary to reestablish microbial populations to maximize the rate of soil stabilization and revegetation.

SUMMARY AND CONCLUSIONS

A bioassay technique for determining microbial response (dehydrogenase activity, microscopal observations, nitrogenase activity, chlorophyll a, and nutrient availability) to amendments of soil systems (mine spoils, tailings, natural soils, etc.) has been developed and utilized to determine requirements for control of terrestrial system imbalances preparatory to beginning revegetation processes. This technique and its application to field analysis of ongoing revegetation techniques should be helpful especially to persons requiring accurate and precise information about needed soil amendments within a shorter time period than is obtained customarily. The conclusions gained from the first phases of this study applied to the Blackbird Minesite in mideastern Idaho are as follows:

I. Field Studies at the Blackbird Mine

1. High temperatures and limited water availability during the working season (July, August) severely limit the ability to revegetate the mine spoil site. Irrigation (sprinkler) will likely be necessary throughout the early revegetation phases.

2. Microbial activity in non-revegetated spoil is essentially nonexistent. Low pH soil (3-5) and heavy metals concentrations are the probable growth inhibiting agents.

3. Revegetation of spoil by the U.S. Forest Service has been accomplished using a comprehensive fertilizer plot design and has resulted in significant increases in microbial populations as measured by dehydrogenase activity.

4. Liming of spoil material produced a significant decrease (to < 1% of original) in soluble heavy metals concentrations (Cu, Fe, Mn), reduced Zn to less than 10% and led to control of spoil pH.

5. Further explanation of microbial response required use of the more controlled microbial bioassays.

II. Microbial Bioassays of Blackbird Mine Spoils

1. Limiting factors for microbial growth in Blackbird Mine spoils were determined to be pH control, soil moisture, and nutrients. Water was not limiting in the bioassays because it was added to all samples at a constant and near saturating level.

2. Control of pH through lime addition resulted in removal of metals and allowed significant increases in microbial activity. In the first bioassay estimates of lime necessary for pH control were too low; lime additions were increased and microbial growth obtained on amended spoil samples was equivalent to control soils (natural forest subsoil from the Blackbird area).

3. Because of continuing oxidation of sulfide minerals in spoil materials, a total lime requirement needs to be determined at least for the upper spoil layers.

4. Because of fixation of nitrogen by blue-green algae located on the soil surface nitrogen was not limiting. Significant amounts of nitrogen were added to the spoil by fixation as estimated by nitrogenase measurements.

5. Optimum nitrogenase activity was observed on spoil samples amended with lime and phosphorus. Calculations indicated that 10.7 lbs. of nitrogen/acre (12 kg/ha) would be added annually to the spoil by blue-green algal activities.

6. Phosphorus was the limiting nutrient for microbial activities in Blackbird Mine spoils.

7. Addition of nitrogen (NH_4 NO_3) apparently inhibited microbial growth.

8. Addition of sawdust, a chelated heavy metal mix, and yeast extract produced no effects which would be considered as caused by other stimulating factors.

ONGOING AND FUTURE WORK

1. Lime requirement for soil pH control in the Blackbird Mine spoils must be determined in light of the continuing microbial oxidation of sulfide minerals and the resulting production of acid over and above existing acidity. A "total" lime requirement is needed. Ongoing work using peroxide techniques, sulfide analysis techniques, heavy metals techniques, and acid-chromate oxidation techniques indicate that a long term lime requirement can be determined. A separate report is being prepared for publication at this time.

2. Ongoing bioassay work will allow a better definition of the appropriate techniques for microbial analysis of disturbed terrestrial systems.

3. Development of an ATP analysis for microbial characterization is planned.

4. Analysis of results of the nitrogen requirement, the relationship of the bioassay to revegetation experiments, and microbial bioassay relationships for other mine spoil types are planned.

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APPENDIX A

TENTATIVE ALGAL BIOASSAY TECHNIQUE FOR OPTIMIZING THE USE OF MINE SPOIL AMENDMENTS FOR REVEGETATION

GENERAL

Apart from the poor physical matrix associated with mine spoils, toxicities, extreme soil reaction, and nutrient deficiency are the major factors inhibiting the establishment of vegetation. Algae share the requirements for inorganic nutrients and some physiological processes (photosynthesis, etc.) with higher plants. This, combined with their rapid growth potential, makes algae a prime indicator of soil deficiency or fertility. Observation of the relative growths will give a rapid assessment (3-4 weeks) of the nutritional status of the spoil and the optimum quantity and variety of amendments necessary for a healthy vegetative and microbial establishment. A basic knowledge of the physical and chemical properties of the spoil material is sufficient to construct a matrix of various amendments.

SPECIAL MATERIALS AND REAGENTS

1. Algae: Any type of soil algae may be used. Cultures of mixed green and blue-green algae have been used successfully. The use of nitrogen fixing blue-green algae allows the evaluation of the soil material to be tested as to its

ability to support nitrogen fixation, an important microbial process. The use of soil algae isolated from the vicinity of the mine spoil to be treated may give information about the ability of the amended spoil to support natural populations of soil algae. The competitive dominance of one or two species of algae after innoculations of the bioassay with a mixed culture of algae may lend further information as to the properties of the soil (especially nutrient status) when compared to the growth requirements of the various organisms used in the inoculations.

AMENDMENTS

Reagent grade chemicals or commercial brand fertilizers and amendments (of standard use in soil cultivation) can be used. Commercial brand fertilizers have a more direct applicability to the field, but a chemical analysis for purity is advisable to avoid drawing faulty conclusions about growth responses.

NOTE: The small sample size used in the bioassay limits the use of field amendments such as dolomite and treble super phosphate in their natural coarse grain form.

a. Lime:

i) Commercial hydroxide of lime 95%
ii) Reagent grade limes as $CaCO_3$, $Ca(OH)_2$, CaO

b. Phosphorus:

i) Chemical grade phosphate salts, i.e., Na_2HPO_4
ii) Commercial phosphate fertilizer, i.e., treble super phosphate (45% P_2O_5)

c. Nitrogen:

i) Chemical grade inorganic nitrogenous compounds, i.e., NH_4NO_3 and KNO_3
ii) Commercial nitrogen fertilizers without P & K

d. Micronutrients and Chelators:

i) Commercial or reagent grade solutions of micronutrients
ii) Any variety of chelators on the market for use in calcareous soils

e. Potassium: K_2SO_4 or KCl, commercial or reagent grade

f. Mulch:

i) Sawdust
ii) Manure (farm or steer - used also as an organic supply, microbial inoculant, chelator, and nutrient addition)
iii) Humus

g. Containers: Covered sterile petri plates or suitable small containers

h. Spoil Material: Collect a large sample of spoil "typical" of the material to be analyzed, fractionate and use material that will pass a 12 mesh-per-inch screen

i. Soil dressing: Collect and fractionate as for spoil material

PROCEDURE

1. Algal inoculum preparation: At least two weeks in advance of the assay a suitable culture of the selected soil algae must be started in order to assure sufficient algal mass to achieve effective inoculation.

a. Soil algae may be cultured in solid or in liquid medium. Liquid culture is preferred for ease of handling and dispensing. However, many soil algae are difficult to culture in liquid medium. Growth of an algal mat on washed white sand nearly saturated with an inorganic nutrient medium has been used successfully. Media such as triple strength NAAM (EPA, 1971a) or Bristol's Solution (Starr, 1964) are recommended.

b. The inoculum culture may be started from algal cultures maintained in the laboratory in liquid or in solid media, or by addition of soil known to contain the desired algae.

c. If the inoculum is grown on sand, it will be necessary to add distilled water occasionally to the culture to maintain near water saturation. The culture must be vented to the atmosphere to allow gas exchange and water will be lost by evaporation.

d. Light and temperature should be maintained at the same conditions as the bioassay (24 ± 2 C, 2100 ± 10% lux).

e. Algal growth should be continued until the chlorophyll a concentrations of the dry sand (top 5 mm) is about 100 μg/g (heavy growth).

f. When growth is complete on the sand, the inoculum should be scraped from the surface (5mm depth), air dried in the dark, and stored for not more than 48 hours in a dark refrigerator (4-6 C).

g. Chlorophyll a concentration of the room dry sand (or liquid culture) should be determined. Inoculum should be added to the soil so that about 50 μg of chlorophyll a are applied 50 g soil.

2. Recommended preliminary analyses of spoil material are as follows: pH, dehydrogenase, chlorophyll a, total nitrogen, total phosphorus, electrical conductivity, and moisture content at saturation (field capacity).

3. Amendments such as lime or lime products, manure or other materials which are to be added dry to all or most of the various treatments being tested should be added in bulk at the desired level to as much soil as will be required for the tests (50 g each) and thoroughly mixed with the soil.

4. 50 g portions of the screened soil material (with common amendments added as per 3 above) are placed in standard 100 x 15 mm petri dish bottoms and spread evenly over the surface of the dish bottom. Three or more replicate assays of each amendment are recommended. Note: If large (> 1% total weight) amounts of dry amendments are added to samples used in the assays, sample weight should be increased to compensate for the amendment.

5. Where possible, amendments should be dissolved in water so that they may be added evenly to the soil in a small volume with the first

watering of the soil. Care must be taken not to exceed saturation (field capacity) of the soil.

6. Various amendments:

   a. Adjustment of pH: Mine spoils often exhibit extreme pH reaction. Oxidation of sulfide minerals can cause severe acidity in some mine spoils. Other spoils are very alkaline. The addition of lime or lime products to acid soils should be based on an estimate of total potential acid production (EPA, 1971b). Flower of sulfur or ferrous sulfate may be added to alkaline soils to lower the pH (Buckman and Brady, 1969). The requirement for sulfur addition may have to be empirically determined in advance.

   b. Phosphorus addition: Phosphate fertilizers or reagent grade phosphate salts should be added at levels that approximate the phosphorus content of fertile soils in the area of the mine spoil revegetation work. If large amounts of lime are required to control soil pH, several levels of phosphorus addition above a known value for fertility should be tried in order to evaluate the immobilization of phosphate by precipitation with the lime.

   c. Nitrogen addition: Nitrogen addition at a level to increase the nitrogen content of the soil to a level of known local fertility should be evaulated. Note: Blue-green algae have not been

observed to respond well to nitrogen addition in bioassays to date. Further investigation of the implications of this phenomena are in progress. Also, the oxidation of NH_4NO_3 has been known to lower soil pH values.

d. Chelator addition: Trace metal nutrients may be made more available by addition of chelating compounds. For soils that are calcareous or have had lime added chelators should be chosen which are not easily saturated by calcium (Wallace, 1956). A mixture of three chelator compounds is recommended. They are as follows:

Abbreviation	Chemical Name	Trade Name*
NaFeDTPA	Mono sodium ferric diethylene-triamine pentaacetate	Sequestrene 330 Fe
DTPA	Diethylenetriamine pentaacetic acid	Chel 330
HFeEDDHA	Ferric chelate of an aromatic phenolic polyamino-carboxylic acid (identity not released)	Chel 138

*Products of Geigy Chemical Company, New York, N. Y.

e. Mulch addition: Manure, peat, wood fibre, etc. help improve the physical matrix of the soil as well as adding nutrients. Addition of these materials at about 2% by weight may improve growth.

f. Other amendments: Addition of other nutrients such as potassium and trace metals may be evaluated. Any amendment that may be thought to improve the fertility or structure of the soil material may be used. Reasonable additions must be used.

7. Inoculation: Add the inoculum to the surface of the soil in the petri dish such that about 50 μg of algal chlorophyll a is added. Liquid inoculum may be carefully pipetted onto the surface in a small volume so that the soil is not saturated (including other liquid additions). White sand with algae attached and mixed with it may be sprinkled on by weight so that sufficient algae are added. Use of more than 0.5 g of sand is not recommended.

8. Physical conditions:

   a. Light intensity = 2100 lux ± 10%

   b. Temperature = 24 ± 2 C

   c. Moisture = Variable. Moisture content can be easily maintained by adding distilled water daily or every other day to the petri dish to bring it to a known weight which corresponds to the desired moisture content. Note: Total soil water potential (psycrometitrically determined) is more critical in regulating algal growth than moisture content. However, if the effects of moisture are not being evaluated a moisture content slightly below saturation is recommended.

9. Monitoring:

a. An easily obtained record of algal growth can be obtained by periodically photographing the surface of the petri dishes in each treatment with the covers removed.

b. Other chemical or biological tests may be performed during the course of the bioassay by sacrificing "extra" replicates of each treatment. Non-destructive measurements such as acetylene reduction for nitrogen fixation may also be performed.

c. At the conclusion of the bioassay (3-4 weeks) all the replicates are analyzed for pertinent parameters individually and the mean values of the various treatments compared to determine the effectiveness of a given treatment.

d. Findings of one bioassay may be used to dictate the need for further testing of various treatments possibly at different levels in subsequent bioassays. Results can be used to screen amendments preparatory to higher plant fertilization studies.

APPENDIX B

METHODS FOR CHARACTERIZING SOIL MICROBIAL COMMUNITIES

I. CHLOROPHYLL A CONTENT (CORRECTED)

GENERAL

1. References: Standard Methods (APHA, 1971), pp. 748-749.
   EPA, 1973.
   Vogelsberg (1975) pp. 9-11.

2. Outline of Method: A known weight of soil as collected (not dried) is extracted in a minimum volume of 90% acetone for 48 hours in the dark under refrigeration with occasional agitation. The chlorophyll-acetone solution is then separated from the soil by filtration settling or centrifugation and analyzed for concentration of chlorophyll a in the presence of pheophytin a by fluorescence or visible spectrophotometry. The sample should be preserved by refrigeration in the dark. Analysis should be done as soon as possible after collection of the sample - preferrably less than 24 hours.

SPECIAL REAGENTS

1. Hydrochloric acid: Concentrated analytical reagent grade hydrochloric acid.

2. 90% acetone: Add 100 ml distilled water to 900 ml analytical reagent grade acetone. Store in a tightly sealing container. Discard if cloudy or colored.

PROCEDURE

1. Place a known weight of soil (usually 1-10 g of field sample) in a 125 ml erlynmeyer flask or large culture tube (large enought to allow for easy mixing by swirling). A subsample can be dried and characterized for dry weight and area conversion of chlorophyll results. Add a known volume of 90% acetone large enough to saturate the soil and allow at least 20 ml of free solution (usually 25-50 ml) and swirl to mix. Seal tightly with a rubber stopper or cork and place in a dark refrigerator (3-6 C). It is imperative to swirl the soil extraction to mix once or twice a day over the 48 hour extraction period. Also, mix just prior to measuring chorophyll.
2. The chlorophyll containing acetone should be separated from the soil by settling and decantation, centrifugation, or filtration. Decantation is preferred (due to time savings) if the operation allows the collection of at least 10 ml of clear solution, and if the analysis is to be done by fluorescence spectrophatometry which has low sensitivity to turbidity. Centrifugation is best for removing turbidity. Filtration may be used, but care should be taken to avoid significant loss of acetone under vacuum.
3. The clear solution may be read for chlorophyll a content in the presence of pheophytin a in the visible spectrophotometer as is described in Standard Methods (APHA, 1971):

Adjust the solvent volume so that the optical density falls between 0.2 and 0.5. Avoid unnecessary dilution. Read the OD at 665 and 750 mμ before and after acidifying with 0.02 ml of concentrated HCl. Subtract the 750 mμ values from the 665 mμ readings in each pair. The corrected 665 mμ readings are then used to calculate the ratio and concentration of chlorophyll and pheophytin in the sample.

Chlorophyll a (C) and pheophytin a (P) per gram of soil are calculated as follows:

$$C(mg/g) = \frac{26.73(665_b - 665_a) \times V}{W}$$

$$P(mg/g) = \frac{26.73\,[1.7\,(665_a) - 665_b] \times U}{W}$$

where 665_b and 665_a are the corrected optical densities of the 90% acetone extract before and after acidification respectively, using a 1 cm light path, V = volume of the extract in liters; W = weight of the soil sample in grams.

4. Fluorescence spectrophotometry is preferred when pigment concentrations are low and turbidity or other interferences exist. Any filter or grating fluorescence spectrophotometer may be used capable of providing excitation spectra of 430 nm (Corning CS 5-60 filter) and reading emission spectra of 663 nm (Corning CS 2-60 filter). The sample is placed in a cuvette and fluorescence recorded. The sample is then acidified with 0.02 ml of concentrated HCl, allowed to stand 30 seconds and fluorescence is read again. The difference between fluorescence before and after acidification is used in the calculations of chlorophyll a concentration.

CALIBRATION OF THE FLUOROMETER AND CALCULATIONS

1. Chlorophyll is extracted from a log-phase aqueous culture of algae by filtration on glass fibre paper and extraction of the filter and attached algae in 90% acetone is done as described under procedure 1 above. The concentration of chlorophyll a (corrected for pheophytin a) in this extract is determined using the visible spectrophotometric method described above.

2. Appropriate dilutions of the known chlorophyll a solution are then read for fluorescence corrected for pheophytin a, and used to calculate the slope of the calibration curve for each sensitivity setting on the fluorometer. That is

$$F_s = \frac{\text{concentration chlorophyll a (mg/l)}}{\text{Fluorometer reading}}$$

where F_s = the slope factor for each sensitivity setting (s).

3. Calculation of chlorophyll a is done as follows

$$\text{Chlorophyll a (mg/g)} = \frac{(f_b - f_a) \times F_s \times V}{W}$$

where f_b and f_a = fluorescence before and after acidification respectively, F_s = slope factor (determined above) for each sensitivity (s), V = extract volume in liters, and W = weight of the sample in grams. Note: Chlorophyll a fluorescence has been found not to be linear above 80 units on the readout dial on the Turner® model 110 fluorometer in our laboratory.

II. FLUORESCENT MICROSCOPY OF SOIL SAMPLES

GENERAL

1. References: F. W. Pauli (1967)

   V. T. Tchan (1953)

2. Outline of Method: A known weight of soil is spread thinly across a known area of a microscope slide in a medium that when dry will cause the soil to adhere to the slide. The slide is then examined by epifluorscent microscopy and the number of fluorescing algae counted in a representative number of randomly selected microscope fields of known area. The number of algae in the total area of the microscope fields in then extrapolated to the number of algae in the weight of soil covering the known area on the slide and hence to the number per gram of soil.

SPECIAL REAGENTS

1. Adhesive material: Methocel - mix 2.5 g methyl cellulose with 90 ml boiling water. Allow to stand for 20 minutes, then place in refrigerator to cool until consistancy of honey.

PROCEDURE

1. Place a drop of methocel in the center of a standard microscope slide.
2. Place 50 to 150 mg room dry freshly collected soil in the drop of methocel, mix, and spread very thinly over a predetermined area of the slide and allow to dry at room temperature in a warm (35 C) incubator.

3. Using epi-fluorescence with excitation illumination having an optimum near 430 nm and an optical barium filter with a sharp cut off near 530 mm, count at least 10 fields of known area for red fluorescing algae. The chlorophyll contained in the cells of the algae fluoresces red under 430 nm illumination.

4. Calculate the number of fluorescing algae per gram as follows

$$\frac{\text{Number of algae}}{\text{g}} = \frac{\dfrac{\underline{a}\ \text{area}\ (\text{cm}^2)}{\underline{b}\ \text{sample weight (g)}}}{\underline{c}\ \text{area of microscope field}} \times \frac{\underline{d}\ \text{total algae counted}}{\underline{e}\ \text{number of fields examined}}$$

which reduces to

$$\frac{\text{Number of algae}}{\text{g}} = \frac{\underline{a}\ \text{cm}^2}{\underline{b}\ \text{g} \cdot \underline{c}\ \text{cm}^2} \times \frac{\underline{d}}{\underline{e}}$$

III. DEHYDROGENASE ACTIVITY

GENERAL

1. References: L. E. Casida, et al., (1964)

   J. J. Skujins, (1967).

2. Outline of Method: A sample of soil (usually 10 g) is incubated with 2, 3, 5 tripheyltetrazolium chloride in aqueous solution for 24 hours after which the reduced product (2, 3, 5 triphenyltetrozolium formazan) is extracted from the soil with methanol and its concentration in the extract is measured colorimetrically. Dehydrogenases catalyze the reaction $XH_2 + A \rightarrow X + AH_2$ in the respiration process, and the method described reflects the respiratory activity of the total microbial community in the soil.

SPECIAL REAGENTS

1. 2, 3, 5 triphenyltetrazolium chloride (TTC) 3%:

   Dissolve 3 g of the chloride in 100 ml of distilled water. Sterilize by filtration.

2. 2, 3, 5 triphenyltetrazolium formazan (formazan) standard. Dissolve not more than 3 mg (ground to a power) of the formazan in 100 ml of reagent grade absolute methanol. Solution may require several hours. Make appropriate dilutions to form a standard curve of adsorbance vs. concentration.

3. Methanol: Reagent grade absolute methanol

PROCEDURE

1. Weigh a sample of freshly collected soil (usually 10 g) and place in one of two or three 16 x 150 mm culture tubes to be sterilized as a control. Cover the tubes with standard culture tube closures.
2. Sterilize the sample in the tube and enough extra empty tubes to give the desired number of replicates by autoclaving 60 minutes at 121 C and 15 psi.
3. When the autoclaved culture tubes have cooled, weigh replicate samples into the empty, sterile tubes. Add 1 ml of 3% TTC and enough seterite distilled water to saturate the soil and leave at least 5 mm depth of free water standing above the soil after mixing and settling (usually 3-5 ml). The exact same amount of sterile water should be added to all replicates.
4. Mix the saturated soil well with a glass rod, close and incubate at 35 ± 1C for 24 hours. Note: Incubation temperatures of 30-37 C are acceptable but all tests should be done at one given temperature controlled to ± 1C, and the incubation temperature reported.
5. After incubation the sample is washed from the culture tube into a buchner funnel using qualitative filter paper with methanol, and the soil is extracted on the funnel with methanol until the orange-red color of the formazan can no longer be seen coming from the soil (usually 50 to 90 ml of methanol required). Make the exact volume up to 100 ml in a 100 ml volumetric flask and save in a tightly sealed flask or bottle.

6. If turbidity can be seen in the methanol extract it should be removed be centrifugation being careful to avoid evaporation. After being clarified, the optical density (OD) of the sample is read in the spectrophotometer at 485 nm with a methanol reference using either a 1 or 5 cm light path.

STANDARDIZATION

1. Make serial dilutions of a known concentration of formazan in methanol until the visible color is almost extinguished.
2. Read and record the OD of each of these solutions on a 1 and 5 cm light path cuvette and construct a standard curve of OD vs. concentration for each light path.
3. The validity of the standard curve should be checked on each analysis date with a known concentration of the formazan.
4. Calculate the dehydrogenase activity (formazan produced) as follows:

$$\mu g \text{ formazan/g} = \frac{1}{\text{standard slope}} \times \frac{a}{} \text{ OD units} \times \frac{100 \text{ vol.}}{b \text{ g sample}}$$

where the standard slope = the OD units per μg formazan per 100 ml.

IV. NITROGEN FIXATION (ACETYLENE REDUCTION)

GENERAL

1. Reference: Hardy et al. (1973)
   Stewart et al. (1967)
   Sorensen (1975)

2. Outline of Method: A basically "intact" sample of surface soil is placed in a petri dish bottom and transported to the laboratory. There it is placed in the special chamber described in Figure 4 below. The natural atmosphere is replaced with an argon based atmosphere to eliminate competition for the nitrogen fixing enzyme system, nitrogenase. An artificial substrate, acetylene ($HC \equiv CH$) is introduced to the atmosphere and is reduced to ethylene ($H_2C = CH_2$) in a molar ratio three times that of N_2 ($N \equiv N$) being converted to $2NH_3$. Ethylene is detected by gas chromatography using flame ionization detection. The detection limit for ethylene is about twenty picomoles (10^{-12} moles). Gas samples are drawn from the incubation vessel atmosphere at regular intervals, and the increase in ethylene content with time converted to nitrogen fixation rate.

SPECIAL REAGENTS

1. Argon atmosphere: Commercially prepared atmosphere containing 0.04% CO_2, 22% O_2, and the balance argon (Matheson Gas Products).
2. Acetylene: Purified acetylene (Matheson Gas Products).
3. Ethylene standard: Ethylene 5 ppm in N_2 (Matheson Gas Products).

PROCEDURE

1. Place the petri dish containing the soil sample on the base of the incubation chamber and bolt the cover in place.

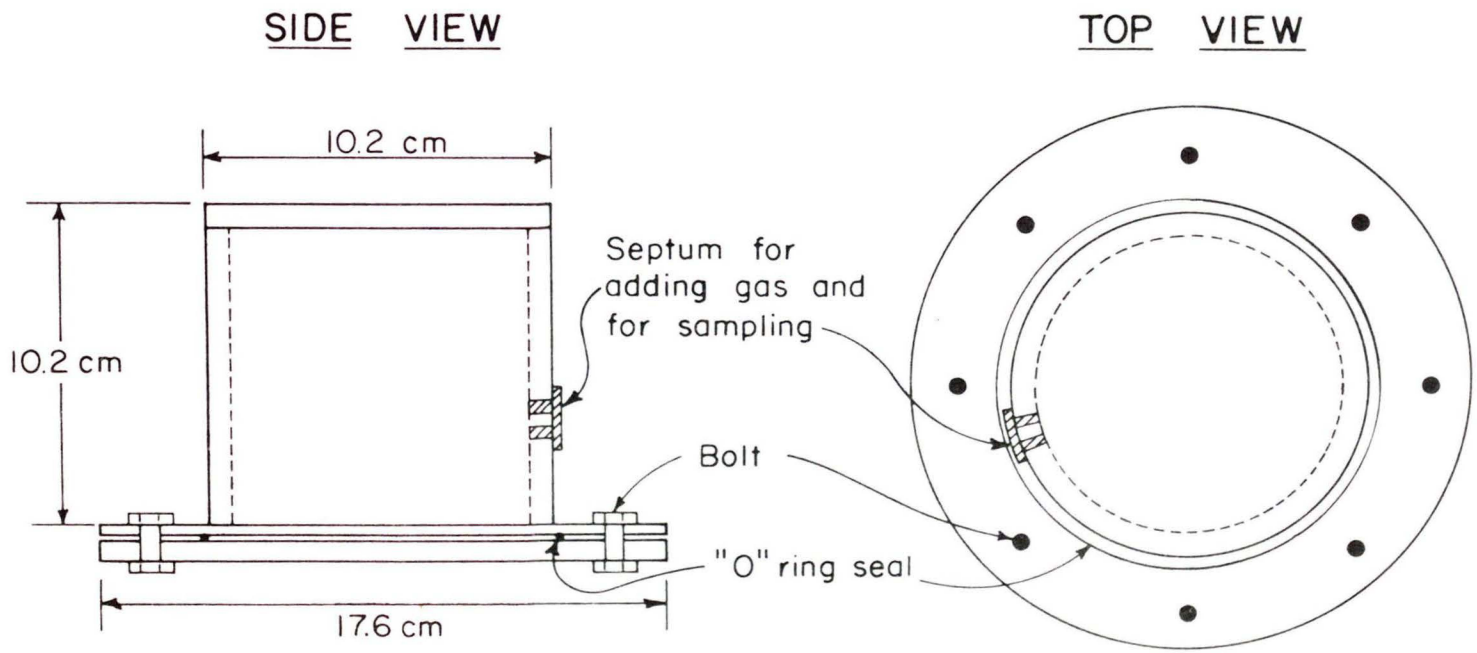

Figure 4. Lucite chamber used in the acetylene-ethylene assay for nitrogenase activity (Nitrogen fixation).

2. Replace the atmosphere with the argon atmosphere by flushing for 1-2 minutes using a flow rate sufficient to replace the volume of the vessel atmosphere three times. Insert a hypodermic needle through which the gas is injected through the septum and allow the gas to escape around the edges of the loose septum.

3. Seal the septum tightly into the incubation chamber body and inject 0.02 atmospheres of acetylene. Equalize the pressure by inserting another hypodermic needle momentarily.

4. Remove samples from the chamber at 10-30 minute intervals, depending on the expected rate, with a hypodermic syringe (or gas tight syringe if direct injection is used).

5. Introduce the atmospheric sample onto the gas chromatograph column through a gas sampling valve if available, being sure to use enough sample to flush and fill the sample loop in the sampling valve. Double or triple injuction technique is possible with the conditions below. Direct injection may also be used, but is less reproducible. A sample size of 0.5 cc is recommended.

6. Gas chromatograph conditions are as follows:

column: 10 ft x 1/8 inches
liquid phase: N', N' -dimethyloxalomide (EDO-1, Supelco) 20%
support: Chromasorb P, 100/120 mesh (Supelco)
carrier gas: He, 30 cc/min
detector: Flame
column temperature: 0 C (ice bath)

STANDARDIZATION

1. Flame ionization response to ethylene concentration has been shown to be linear over several orders of magnitude. Therefore, the response to a single concentration standard is all that is necessary for calibration. Peak height or peak may be used as the indicator of concentration of ethylene.

$$\frac{\text{moles } N_2 \text{ fixed}}{[\text{min}]\,[\text{area (or weight) of sample}]} = \frac{\text{moles } C_2H_4}{\text{integration unit}} \times \frac{\text{integration units}}{\text{sample}} \times$$

$$\frac{\underline{a} \text{ cc}}{\text{incubation chamber}} \div \underline{b} \text{ vol. of sample (cc)} \times \frac{1 \text{ mole } N_2 \text{ fixed}}{3 \text{ mole } C_2H_4} \times \frac{1}{\text{lapsed time (minutes)}}$$

V. TOTAL NITROGEN CONTENT

GENERAL

1. Reference: Coleman Instruments (1968)
   Sorensen (1975)
2. Outline of method: Micro-Dumas total nitrogen is determined from a known weight (50-200 mg) of a prepared soil material in the Coleman® model 29 nitrogen analyzer.

PROCEDURE

1. The soil sample should be dried to constant weight at 103-105 C and allowed to equilibrate with a desicator atmosphere.

2. The sample is thoroughly mixed, and a 50 to 200 mg subsample is subjected to pyrolysis in the nitrogen analyzer. The nitrogen produced is measured volumetrically and the percentage nitrogen content is calculated according to instrument instructions.

VI. PHOSPHORUS CONTENTS

GENERAL

1. References: Bureau of Reclamation (1967)

   Olsen et al., (1954)

   Strickland and Parsons (1968)

2. Outline of Method: Reactive orthophosphate is extracted by agitation with pH 8.5 bicarbonate buffer. The extracted phosphate is then determined by the ascorbic acid or Murphy Riley Technique (Strickland and Parsons, 1968; APHA, 1971).

SPECIAL REAGENTS

1. Sodium bicarbonate solution (0.5 M, pH 8.5): Dissolve 42 g of reagent grade $NaHCO_3$ in 500 ml of distilled water (DW) and dilute to 1 l with DW. Adjust the pH of the solution to pH 8.5 using concentrated NaOH.

2. Armstrong Reagent: Add 122 ml of conc. H_2SO_4 to 800 ml of DW. While the solution is still hot, add 10.5 g of Ammonium Molybdate and 0.3 g Antimony potassium tartrate. Heat to dissolve; cool, and dilute to exactly 1 liter with DW, stable indefinitely.

3. Ascorbic Acid Solution: Dissolve 3 g of Ascorbic Acid in 100 ml of DW. Note: Stable only one week (store in refrigerator).

PROCEDURE

1. Place 5 g of soil in an erlynmeyer flask and add 100 ml of sodium bicarbonate solution. Mix and place on an orbital shaker at constant speed (fast enough to mix the soil) for 30 minutes.
2. The soil suspension is then centrifuged and five ml of the supernatant is diluted to 50 ml for ortho phosphate determination.
3. To the 50 ml of diluted sample add 5 ml of Armstrong Reagent, and 1 ml of Ascorbic Acid; swirl to mix.
4. Allow a 20 min. reaction period (stable up to 2 hrs.) and then read the absorbance against DW at 885 nm using a 1 or 5 cm cell.
5. Subtract turbidity correction (if any) from sample absorbance; plug this corrected absorbance into the slope-intercept formula for the standard curve to obtain μg PO_4-P/l.

STANDARDIZATION

1. Dilute a 1 mg PO_4-P/ml K_2HPO_4 stock standard solution to concentrations ranging from 0 to 700 μg P/l.
2. Construct a standard curve of adsorbance at 885 nm and compute the slope.

APPENDIX C

DATA AND STATISTICAL ANALYSIS OF DATA FROM BIOASSAYS 1 AND 2

Table 8. Data from Bioassay 1 including means, standard deviations, and coefficients of variations.

1 DESERT CONTROL

REPLICATES	PH	TOTAL N MG/G	N FIXATION MG-N/HA-MIN	CHL A MG/G	DEHYDROGENASE MG-FORMAZAN/G	EXTRACTABLE P UG/G
1	8.0	1.30	9.	.1940		
2	8.0	1.40	2.	.1940		
3	8.1	1.30	38.	.2120		
MEAN	8.03	1.33	16.33	.2000	0.1160	0.00
STD DEVIATION	0.00	0.06	19.09	0.0104		
COEF VARIATION	0.00	0.04	1.17	0.0520		

2 SUBSOIL CONTROL

REPLICATES	PH	TOTAL N MG/G	N FIXATION MG-N/HA-MIN	CHL A MG/G	DEHYDROGENASE MG-FORMAZAN/G	EXTRACTABLE P UG/G
1	5.8	0.12	9.	.0160		
2	6.0	0.12	10.	.0340		
3	5.9	0.11	10.	.0340		
MEAN	5.89	0.13	9.67	.0280	0.0090	1.70
STD DEVIATION	0.00	0.03	0.58	0.0104		
COEF VARIATION	0.00	0.24	0.06	0.3712		

3 SUBSOIL + 15MG LIME, 179MG AMMONIUM NITRATE

REPLICATES	PH	TOTAL N MG/G	N FIXATION MG-N/HA-MIN	CHL A MG/G	DEHYDROGENASE MG-FORMAZAN/G	EXTRACTABLE P UG/G
1	5.6	1.10	-0.	.1410		
2	5.7	1.10	-0.	.1240		
3	5.7	1.20	-0.	.1240		
MEAN	5.66	1.13	0.05	.1297	0.0020	0.00
STD DEVIATION	0.00	0.06	0.00	0.0098		
COEF VARIATION	0.00	0.05	0.00	0.0757		

Table 8. Continued.

4 SUBSOIL + 15MG LIME, 4.4MG TREBLE SUPER PHOSPHATE

REPLICATES	PH	TOTAL N MG/G	N FIXATION MG-N/HA-MIN	CHL A MG/G	DEHYDROGENASE MG-FORMAZAN/G	EXTRACTABLE P UG/G
1	6.8	0.26	87.	.2470		
2	6.7	0.29	84.	.2300		
3	6.7	0.29	102.	.3000		
MEAN	6.73	0.28	91.00	.2590	0.0500	4.70
STD DEVIATION	0.00	0.02	9.64	0.0365		
COEF VARIATION	0.00	0.06	0.11	0.1410		

5 SUBSOIL + 15MG LIME

REPLICATES	PH	TOTAL N MG/G	N FIXATION MG-N/HA-MIN	CHL A MG/G	DEHYDROGENASE MG-FORMAZAN/G	EXTRACTABLE P UG/G
1	6.9	0.10	47.	.0900		
2	6.8	0.10	49.	.1350		
3	7.0	0.19	83.	.1410		
MEAN	6.89	0.13	59.67	.1220	0.0190	0.00
STD DEVIATION	0.00	0.05	20.23	0.0279		
COEF VARIATION	0.00	0.40	0.34	0.2285		

6 SUBSOIL + 15MG LIME, 125MG YEAST

REPLICATES	PH	TOTAL N MG/G	N FIXATION MG-N/HA-MIN	CHL A MG/G	DEHYDROGENASE MG-FORMAZAN/G	EXTRACTABLE P UG/G
1	7.1	0.27	88.	.3710		
2	7.0	0.24	73.	.4240		
3	7.3	0.29	80.	.4590		
MEAN	7.12	0.27	80.33	.4180	0.0350	6.50
STD DEVIATION	0.00	0.03	7.51	0.0443		
COEF VARIATION	0.00	0.09	0.09	0.1060		

7 SUBSOIL + 15MG LIME, 2.5G SAWDUST

REPLICATES	PH	TOTAL N MG/G	N FIXATION MG-N/HA-MIN	CHL A MG/G	DEHYDROGENASE MG-FORMAZAN/G	EXTRACTABLE P UG/G
1	6.9	0.21	52.	.0260		
2	6.9	0.14	49.	.0240		
3	7.0	0.10	43.	.0010		
MEAN	6.93	0.15	48.00	.0170	0.0140	0.00
STD DEVIATION	0.00	0.06	4.58	0.0139		
COEF VARIATION	0.00	0.37	0.10	0.8172		

Table 8. Continued.

8 SUBSOIL + 15MG LIME, HEAVY METAL MIX

REPLICATES	PH	TOTAL N MG/G	N FIXATION MG-N/HA-MIN	CHL A MG/G	DEHYDROGENASE MG-FORMAZAN/G	EXTRACTABLE P UG/G
1	6.9	0.13	56.	.2650		
2	6.9	0.18	58.	.4940		
3	7.0	0.15	69.	.2470		
MEAN	6.93	0.15	61.00	.3353	0.0150	0.00
STD DEVIATION	0.00	0.03	7.00	0.1377		
COEF VARIATION	0.00	0.16	0.11	0.4106		

9 SUBSOIL + 15MG LIME, 179MG AMMONIUM NITRATE, 4.4MG TSP, HEAVY METAL MIX

REPLICATES	PH	TOTAL N MG/G	N FIXATION MG-N/HA-MIN	CHL A MG/G	DEHYDROGENASE MG-FORMAZAN/G	EXTRACTABLE P UG/G
1	5.6	1.00	-0.	.3880		
2	5.7	1.10	-0.	.3530		
3	5.8	1.10	-0.	.4240		
MEAN	5.69	1.07	0.05	.3883	0.0020	3.90
STD DEVIATION	0.00	0.06	0.00	0.0355		
COEF VARIATION	0.00	0.05	0.00	0.0914		

10 SUBSOIL + 15MG LIME, 4.4MG TREBLE SUPER PHOSPHATE, HEAVY METAL MIX

REPLICATES	PH	TOTAL N MG/G	N FIXATION MG-N/HA-MIN	CHL A MG/G	DEHYDROGENASE MG-FORMAZAN/G	EXTRACTABLE P UG/G
1	7.0	0.08	73.	.1940		
2	7.0	0.08	108.	.4410		
3	7.0	0.10	96.	.3880		
MEAN	7.00	0.09	92.33	.3410	0.0420	5.10
STD DEVIATION	0.00	0.01	17.79	0.1300		
COEF VARIATION	0.00	0.13	0.19	0.3813		

11 SPOIL CONTROL

REPLICATES	PH	TOTAL N MG/G	N FIXATION MG-N/HA-MIN	CHL A MG/G	DEHYDROGENASE MG-FORMAZAN/G	EXTRACTABLE P UG/G
1	3.8	0.07	0.	.0002		
2	0.0	0.00	0.	.0000		
3	0.0	0.00	0.	.0000		
MEAN	3.80	0.07	0.00	.0002	0.0000	0.00
STD DEVIATION	0.00	0.00	0.00	0.0000		
COEF VARIATION	0.00	0.00	0.00	0.0000		

Table 8. Continued.

12 SPOIL + 100MG LIME, 173MG AMMONIUM NITRATE

REPLICATES	PH	TOTAL N MG/G	N FIXATION MG-N/HA-MIN	CHL A MG/G	DEHYDROGENASE MG-FORMAZAN/G	EXTRACTABLE P UG/G
1	3.8	0.91	0.	.0005		
2	0.0	0.00	0.	.0000		
3	0.0	0.00	0.	.0000		
MEAN	3.80	0.91	0.00	.0005	0.0000	0.00
STD DEVIATION	0.00	0.00	0.00	0.0000		
COEF VARIATION	0.00	0.00	0.00	0.0000		

13 SPOIL + 100MG LIME

REPLICATES	PH	TOTAL N MG/G	N FIXATION MG-N/HA-MIN	CHL A MG/G	DEHYDROGENASE MG-FORMAZAN/G	EXTRACTABLE P UG/G
1	4.3	0.04	0.	.0140		
2	4.4	0.03	0.	.0140		
3	0.0	0.00	0.	.0000		
MEAN	4.35	0.04	0.00	.0140	0.0000	0.00
STD DEVIATION	0.00	0.01	0.00	0.0000		
COEF VARIATION	0.00	0.20	0.00	0.0000		

14 SPOIL + 100MG LIME, 125MG YEAST

REPLICATES	PH	TOTAL N MG/G	N FIXATION MG-N/HA-MIN	CHL A MG/G	DEHYDROGENASE MG-FORMAZAN/G	EXTRACTABLE P UG/G
1	4.5	0.15	0.	.0220		
2	0.0	0.00	0.	.0000		
3	0.0	0.00	0.	.0000		
MEAN	4.50	0.15	0.00	.0220	0.0000	0.00
STD DEVIATION	0.00	0.00	0.00	0.0000		
COEF VARIATION	0.00	0.00	0.00	0.0000		

15 SPOIL + 100MG LIME, 2.5G SAWDUST

REPLICATES	PH	TOTAL N MG/G	N FIXATION MG-N/HA-MIN	CHL A MG/G	DEHYDROGENASE MG-FORMAZAN/G	EXTRACTABLE P UG/G
1	4.8	0.28	0.	.0004		
2	0.0	0.00	0.	.0000		
3	0.0	0.00	0.	.0000		
MEAN	4.80	0.28	0.00	.0004	0.0000	0.00
STD DEVIATION	0.00	0.00	0.00	0.0000		
COEF VARIATION	0.00	0.00	0.00	0.0000		

Table 8. Continued.

16 SPOIL + 100MG LIME, HEAVY METAL MIX

REPLICATES	PH	TOTAL N MG/G	N FIXATION MG-N/HA-MIN	CHL A MG/G	DEHYDROGENASE MG-FORMAZAN/G	EXTRACTABLE P UG/G
1	4.2	0.01	0.	.0100		
2	4.3	0.12	0.	.0080		
3	0.0	0.00	0.	.0000		
MEAN	4.25	0.06	0.00	.0090	0.0000	0.00
STD DEVIATION	0.00	0.08	0.00	0.0014		
COEF VARIATION	0.00	1.20	0.00	0.1571		

17 SPOIL + 100MG LIME, 173MG AMMONIUM NITRATE, HEAVY METAL MIX

REPLICATES	PH	TOTAL N MG/G	N FIXATION MG-N/HA-MIN	CHL A MG/G	DEHYDROGENASE MG-FORMAZAN/G	EXTRACTABLE P UG/G
1	3.8	0.89	0.	.0005		
2	0.0	0.00	0.	.0000		
3	0.0	0.00	0.	.0000		
MEAN	3.80	0.89	0.00	.0005	0.0000	0.00
STD DEVIATION	0.00	0.00	0.00	0.0000		
COEF VARIATION	0.00	0.00	0.00	0.0000		

18 MIX CONTROL

REPLICATES	PH	TOTAL N MG/G	N FIXATION MG-N/HA-MIN	CHL A MG/G	DEHYDROGENASE MG-FORMAZAN/G	EXTRACTABLE P UG/G
1	4.3	0.10	1.	.0050		
2	4.4	0.10	1.	.0090		
3	4.4	0.03	-0.	.0090		
MEAN	4.36	0.08	0.68	.0077	0.0000	12.90
STD DEVIATION	0.00	0.04	0.55	0.0023		
COEF VARIATION	0.00	0.53	0.80	0.3012		

19 MIX + 50MG LIME, 176MG AMMONIUM NITRATE

REPLICATES	PH	TOTAL N MG/G	N FIXATION MG-N/HA-MIN	CHL A MG/G	DEHYDROGENASE MG-FORMAZAN/G	EXTRACTABLE P UG/G
1	4.3	1.10	0.	.0002		
2	4.5	0.90	0.	.0002		
3	4.6	1.10	0.	.0002		
MEAN	4.45	1.03	0.00	.0002	0.0000	0.00
STD DEVIATION	0.00	0.12	0.00	0.0000		
COEF VARIATION	0.00	0.11	0.00	0.0000		

Table 8. Continued.

20 MIX + 50MG LIME, 1.9MG TREBLE SUPER PHOSPHATE

REPLICATES	PH	TOTAL N MG/G	N FIXATION MG-N/HA-MIN	CHL A MG/G	DEHYDROGENASE MG-FORMAZAN/G	EXTRACTABLE P UG/G
1	4.9	0.09	-0.	.0200		
2	0.0	0.12	2.	.0900		
3	0.0	0.16	12.	.2820		
MEAN	4.90	0.14	4.68	.1307	0.0070	15.50
STD DEVIATION	0.00	0.04	6.41	0.1357		
COEF VARIATION	0.00	0.31	1.37	1.0381		

21 MIX + 50MG LIME

REPLICATES	PH	TOTAL N MG/G	N FIXATION MG-N/HA-MIN	CHL A MG/G	DEHYDROGENASE MG-FORMAZAN/G	EXTRACTABLE P UG/G
1	4.9	0.06	-0.	.0450		
2	0.0	0.06	-0.	.0450		
3	0.0	0.04	8.	.3000		
MEAN	4.90	0.05	2.70	.1300	0.0030	0.00
STD DEVIATION	0.00	0.01	4.59	0.1472		
COEF VARIATION	0.00	0.22	1.70	1.1325		

22 MIX + 50MG LIME, 125MG YEAST

REPLICATES	PH	TOTAL N MG/G	N FIXATION MG-N/HA-MIN	CHL A MG/G	DEHYDROGENASE MG-FORMAZAN/G	EXTRACTABLE P UG/G
1	5.4	0.02	2.	.0580		
2	0.0	0.18	3.	.0860		
3	0.0	0.28	1.	.1300		
MEAN	5.40	0.16	2.00	.0913	0.0060	19.40
STD DEVIATION	0.00	0.13	1.00	0.0363		
COEF VARIATION	0.00	0.82	0.50	0.3974		

23 MIX + 50MG LIME, 2.5G SAWDUST

REPLICATES	PH	TOTAL N MG/G	N FIXATION MG-N/HA-MIN	CHL A MG/G	DEHYDROGENASE MG-FORMAZAN/G	EXTRACTABLE P UG/G
1	4.9	0.19	0.	.0007		
2	0.0	0.30	0.	.0005		
3	0.0	0.32	0.	.0004		
MEAN	4.90	0.27	0.00	.0005	0.0000	0.00
STD DEVIATION	0.00	0.07	0.00	0.0002		
COEF VARIATION	0.00	0.26	0.00	0.2864		

Table 8. Continued.

24 MIX + 50MG LIME, HEAVY METAL MIX

REPLICATES	PH	TOTAL N MG/G	N FIXATION MG-N/HA-MIN	CHL A MG/G	DEHYDROGENASE MG-FORMAZAN/G	EXTRACTABLE P UG/G
1	4.9	0.00	0.	.0760		
2	0.0	0.12	0.	.0450		
3	0.0	0.14	0.	.0540		
MEAN	4.90	0.13	0.00	.0583	0.0030	0.00
STD DEVIATION	0.00	0.01	0.00	0.0159		
COEF VARIATION	0.00	0.11	0.00	0.2734		

25 MIX + 50MG LIME, 176MG AMMONIUM NITRATE, 1.9MG TSP, HEAVY METAL MIX

REPLICATES	PH	TOTAL N MG/G	N FIXATION MG-N/HA-MIN	CHL A MG/G	DEHYDROGENASE MG-FORMAZAN/G	EXTRACTABLE P UG/G
1	4.3	1.04	0.	.0002		
2	0.0	1.20	0.	.0003		
3	0.0	0.98	0.	.0002		
MEAN	4.30	1.07	0.00	.0002	0.0000	19.30
STD DEVIATION	0.00	0.11	0.00	0.0001		
COEF VARIATION	0.00	0.11	0.00	0.2474		

26 MIX + 50MG LIME, HEAVY METAL MIX, 1.9MG TREBLE SUPER PHOSPHATE

REPLICATES	PH	TOTAL N MG/G	N FIXATION MG-N/HA-MIN	CHL A MG/G	DEHYDROGENASE MG-FORMAZAN/G	EXTRACTABLE P UG/G
1	5.1	0.08	0.	.0540		
2	0.0	0.02	0.	.0540		
3	0.0	0.05	0.	.1210		
MEAN	5.10	0.05	0.00	.0763	0.0020	18.20
STD DEVIATION	0.00	0.03	0.00	0.0387		
COEF VARIATION	0.00	0.60	0.00	0.5068		

ZERO INDICATES NO READING TAKEN
NEGATIVE VALUES INDICATES NITROGEN FIXATION RATE LESS THAN 0.05

Table 9. Data from Bioassay 2 including means, standard deviations, and coefficients of variation.

1 SUBSOIL CONTROL

REPLICATES	PH	TOTAL N MG/G	N FIXATION MG-N/HA-MIN	CHL A MG/G	DEHYDROGENASE MG-FORMAZAN/G	EXTRACTABLE P UG/G
1	5.8	0.09	53.	.0890		
2	5.9	0.09	39.	.0720		
3	5.9	0.10	35.	.0950		
MEAN	5.86	0.09	42.33	.0853	0.0090	1.66
STD DEVIATION	0.00	0.01	9.45	0.0119		
COEF VARIATION	0.00	0.06	0.22	0.1398		

2 SUBSOIL + 15MG LIME

REPLICATES	PH	TOTAL N MG/G	N FIXATION MG-N/HA-MIN	CHL A MG/G	DEHYDROGENASE MG-FORMAZAN/G	EXTRACTABLE P UG/G
1	6.8	0.17	76.	.1940		
2	6.7	0.23	63.	.1670		
3	6.9	0.07	86.	.1670		
MEAN	6.79	0.16	75.00	.1760	0.0230	1.26
STD DEVIATION	0.00	0.08	11.53	0.0156		
COEF VARIATION	0.00	0.52	0.15	0.0886		

3 SUBSOIL + 200MG CALCIUM CARBONATE

REPLICATES	PH	TOTAL N MG/G	N FIXATION MG-N/HA-MIN	CHL A MG/G	DEHYDROGENASE MG-FORMAZAN/G	EXTRACTABLE P UG/G
1	8.0	0.15	133.	.4770		
2	8.0	0.06	121.	.3000		
3	8.0	0.18	113.	.4410		
MEAN	8.00	0.13	122.33	.4060	0.0310	0.81
STD DEVIATION	0.00	0.06	10.07	0.0935		
COEF VARIATION	0.00	0.48	0.08	0.2304		

Table 9. Continued.

4 SUBSOIL + 15MG LIME, 21.6MG SODIUM MONOHYDROGEN PHOSPHATE

REPLICATES	PH	TOTAL N MG/G	N FIXATION MG-N/HA-MIN	CHL A MG/G	DEHYDROGENASE MG-FORMAZAN/G	EXTRACTABLE P UG/G
1	7.2	0.35	60.	.8470		
2	7.2	0.24	83.	.6000		
3	7.1	0.31	118.	.4590		
MEAN	7.16	0.31	87.00	.6353	0.0710	12.72
STD DEVIATION	0.00	0.04	29.21	0.1964		
COEF VARIATION	0.00	0.13	0.34	0.3091		

5 SUBSOIL + 15MG LIME, 10.8MG SODIUM MONOHYDROGEN PHOSPHATE

REPLICATES	PH	TOTAL N MG/G	N FIXATION MG-N/HA-MIN	CHL A MG/G	DEHYDROGENASE MG-FORMAZAN/G	EXTRACTABLE P UG/G
1	7.0	0.20	117.	.4770		
2	7.0	0.19	109.	.4590		
3	7.1	0.60	148.	.3880		
MEAN	7.03	0.33	124.67	.4413	0.0550	6.30
STD DEVIATION	0.00	0.23	20.60	0.0471		
COEF VARIATION	0.00	0.71	0.17	0.1066		

6 SUBSOIL + 15MG LIME, 21.6MG SODIUM MONOHYDROGEN PHOSPHATE, HEAVY METAL MIX

REPLICATES	PH	TOTAL N MG/G	N FIXATION MG-N/HA-MIN	CHL A MG/G	DEHYDROGENASE MG-FORMAZAN/G	EXTRACTABLE P UG/G
1	7.1	0.26	133.	.7060		
2	7.2	0.39	121.	.7410		
3	7.2	0.55	113.	.6180		
MEAN	7.16	0.40	122.33	.6883	0.0540	9.97
STD DEVIATION	0.00	0.15	10.07	0.0634		
COEF VARIATION	0.00	0.36	0.08	0.0921		

7 SUBSOIL + 15MG LIME, 10.8MG SODIUM MONOHYDROGEN PHOSPHATE, HEAVY METAL MIX

REPLICATES	PH	TOTAL N MG/G	N FIXATION MG-N/HA-MIN	CHL A MG/G	DEHYDROGENASE MG-FORMAZAN/G	EXTRACTABLE P UG/G
1	7.1	0.14	112.	.5300		
2	7.0	0.19	121.	.6530		
3	7.1	0.12	141.	.4410		
MEAN	7.06	0.15	124.67	.5413	0.0840	4.54
STD DEVIATION	0.00	0.04	14.84	0.1065		
COEF VARIATION	0.00	0.24	0.12	0.1967		

Table 9. Continued.

8 SUBSOIL + 15MG LIME, HEAVY METAL MIX

REPLICATES	PH	TOTAL N MG/G	N FIXATION MG-N/HA-MIN	CHL A MG/G	DEHYDROGENASE MG-FORMAZAN/G	EXTRACTABLE P UG/G
1	6.9	0.16	68.	.1940		
2	7.0	0.10	54.	.1770		
3	7.0	0.15	56.	.1770		
MEAN	6.96	0.14	59.33	.1827	0.0290	0.88
STD DEVIATION	0.00	0.03	7.57	0.0098		
COEF VARIATION	0.00	0.24	0.13	0.0537		

9 SUBSOIL + 15MG LIME, 1.1MG TREBLE SUPER PHOSPHATE

REPLICATES	PH	TOTAL N MG/G	N FIXATION MG-N/HA-MIN	CHL A MG/G	DEHYDROGENASE MG-FORMAZAN/G	EXTRACTABLE P UG/G
1	6.9	0.18	81.	.4240		
2	6.6	0.32	113.	.3350		
3	7.1	0.17	99.	.5120		
MEAN	6.82	0.22	97.67	.4237	0.0430	2.75
STD DEVIATION	0.00	0.08	16.04	0.0885		
COEF VARIATION	0.00	0.38	0.16	0.2089		

10 SUBSOIL + 15MG LIME, 2.2MG TREBLE SUPER PHOSPHATE

REPLICATES	PH	TOTAL N MG/G	N FIXATION MG-N/HA-MIN	CHL A MG/G	DEHYDROGENASE MG-FORMAZAN/G	EXTRACTABLE P UG/G
1	7.1	0.18	119.	.4240		
2	7.1	0.09	108.	.5120		
3	7.1	0.13	112.	.5120		
MEAN	7.10	0.13	113.00	.4827	0.0000	0.00
STD DEVIATION	0.00	0.05	5.57	0.0508		
COEF VARIATION	0.00	0.34	0.05	0.1053		

11 SUBSOIL + 15MG LIME, 3.3MG TREBLE SUPER PHOSPHATE

REPLICATES	PH	TOTAL N MG/G	N FIXATION MG-N/HA-MIN	CHL A MG/G	DEHYDROGENASE MG-FORMAZAN/G	EXTRACTABLE P UG/G
1	6.9	0.14	0.	.4770		
2	7.0	0.23	0.	.3350		
3	7.0	0.17	0.	.5300		
MEAN	6.96	0.18	0.00	.4473	0.0480	3.38
STD DEVIATION	0.00	0.05	0.00	0.1008		
COEF VARIATION	0.00	0.25	0.00	0.2254		

Table 9. Continued.

12 SUBSOIL + 15MG LIME, 4.4MG TREBLE SUPER PHOSPHATE

REPLICATES	PH	TOTAL N MG/G	N FIXATION MG-N/HA-MIN	CHL A MG/G	DEHYDROGENASE MG-FORMAZAN/G	EXTRACTABLE P UG/G
1	7.1	0.11	119.	.5830		
2	7.1	0.23	87.	.5120		
3	7.0	0.19	129.	.6000		
MEAN	7.06	0.18	111.67	.5650	0.0550	5.27
STD DEVIATION	0.00	0.06	21.94	0.0467		
COEF VARIATION	0.00	0.35	0.20	0.0826		

13 SUBSOIL + 15MG LIME, 5.5MG TREBLE SUPER PHOSPHATE

REPLICATES	PH	TOTAL N MG/G	N FIXATION MG-N/HA-MIN	CHL A MG/G	DEHYDROGENASE MG-FORMAZAN/G	EXTRACTABLE P UG/G
1	7.0	0.13	0.	.4410		
2	7.0	0.25	0.	.5120		
3	7.0	0.26	0.	.6530		
MEAN	7.00	0.21	0.00	.5353	0.0410	6.53
STD DEVIATION	0.00	0.07	0.00	0.1079		
COEF VARIATION	0.00	0.34	0.00	0.2016		

14 SUBSOIL + 15MG LIME, 6.6MG TREBLE SUPER PHOSPHATE

REPLICATES	PH	TOTAL N MG/G	N FIXATION MG-N/HA-MIN	CHL A MG/G	DEHYDROGENASE MG-FORMAZAN/G	EXTRACTABLE P UG/G
1	7.0	0.22	104.	.4410		
2	7.0	0.12	107.	.4240		
3	7.0	0.16	127.	.6710		
MEAN	7.00	0.17	112.67	.5120	0.0420	7.11
STD DEVIATION	0.00	0.05	12.50	0.1380		
COEF VARIATION	0.00	0.30	0.11	0.2695		

15 SPOIL CONTROL

REPLICATES	PH	TOTAL N MG/G	N FIXATION MG-N/HA-MIN	CHL A MG/G	DEHYDROGENASE MG-FORMAZAN/G	EXTRACTABLE P UG/G
1	3.7	0.02	0.	.0030		
2	3.6	0.03	0.	.0020		
3	3.6	0.05	0.	.0020		
MEAN	3.63	0.05	0.00	.0023	0.0000	23.48
STD DEVIATION	0.00	0.02	0.00	0.0006		
COEF VARIATION	0.00	0.40	0.00	0.2474		

Table 9. Continued.

16 SPOIL + 350MG LIME

REPLICATES	PH	TOTAL N MG/G	N FIXATION MG-N/HA-MIN	CHL A MG/G	DEHYDROGENASE MG-FORMAZAN/G	EXTRACTABLE P UG/G
1	7.5	0.13	175.	.4940		
2	7.5	0.16	162.	.3880		
3	7.6	0.24	180.	.4240		
MEAN	7.53	0.18	172.33	.4353	0.0120	39.13
STD DEVIATION	0.00	0.06	9.29	0.0539		
COEF VARIATION	0.00	0.32	0.05	0.1238		

17 SPOIL + 8000MG CALCIUM CARBONATE

REPLICATES	PH	TOTAL N MG/G	N FIXATION MG-N/HA-MIN	CHL A MG/G	DEHYDROGENASE MG-FORMAZAN/G	EXTRACTABLE P UG/G
1	7.6	0.22	140.	.4060		
2	7.6	0.32	121.	.4760		
3	7.6	0.51	146.	.2820		
MEAN	7.60	0.35	135.67	.3880	0.0110	28.41
STD DEVIATION	0.00	0.15	13.05	0.0982		
COEF VARIATION	0.00	0.42	0.10	0.2532		

18 SPOIL + 350MG LIME, 10.8MG SODIUM MONOHYDROGEN PHOSPHATE

REPLICATES	PH	TOTAL N MG/G	N FIXATION MG-N/HA-MIN	CHL A MG/G	DEHYDROGENASE MG-FORMAZAN/G	EXTRACTABLE P UG/G
1	7.6	0.17	234.	.4240		
2	7.6	0.21	231.	.3710		
3	7.6	0.13	215.	.3530		
MEAN	7.60	0.17	226.67	.3827	0.0130	46.09
STD DEVIATION	0.00	0.04	10.21	0.0369		
COEF VARIATION	0.00	0.24	0.05	0.0965		

19 SPOIL + 350MG LIME, 10.8MG SODIUM MONOHYDROGEN PHOSPHATE, HEAVY METAL MIX

REPLICATES	PH	TOTAL N MG/G	N FIXATION MG-N/HA-MIN	CHL A MG/G	DEHYDROGENASE MG-FORMAZAN/G	EXTRACTABLE P UG/G
1	7.7	0.11	210.	.3180		
2	7.6	0.23	226.	.4940		
3	7.7	0.26	265.	.3530		
MEAN	7.66	0.20	233.67	.3883	0.0180	0.00
STD DEVIATION	0.00	0.08	28.29	0.0932		
COEF VARIATION	0.00	0.40	0.12	0.2399		

Table 9. Continued.

20 SPOIL + 350MG LIME, HEAVY METAL MIX

REPLICATES	PH	TOTAL N MG/G	N FIXATION MG-N/HA-MIN	CHL A MG/G	DEHYDROGENASE MG-FORMAZAN/G	EXTRACTABLE P UG/G
1	7.7	0.17	181.	.2470		
2	7.7	0.11	163.	.3710		
3	7.7	0.19	162.	.1940		
MEAN	7.70	0.16	170.33	.2707	0.0020	0.00
STD DEVIATION	0.00	0.04	9.45	0.0908		
COEF VARIATION	0.00	0.27	0.06	0.3356		

ZERO INDICATES NO READING TAKEN
NEGATIVE VALUES INDICATES NITROGEN FIXATION RATE LESS THAN 0.05

Table 10. T-test statistical comparison of nitrogen fixation analyses from Bioassay 1. Treatments with insignificant difference are blocked out. (See Figure 2 for treatment number identification.)

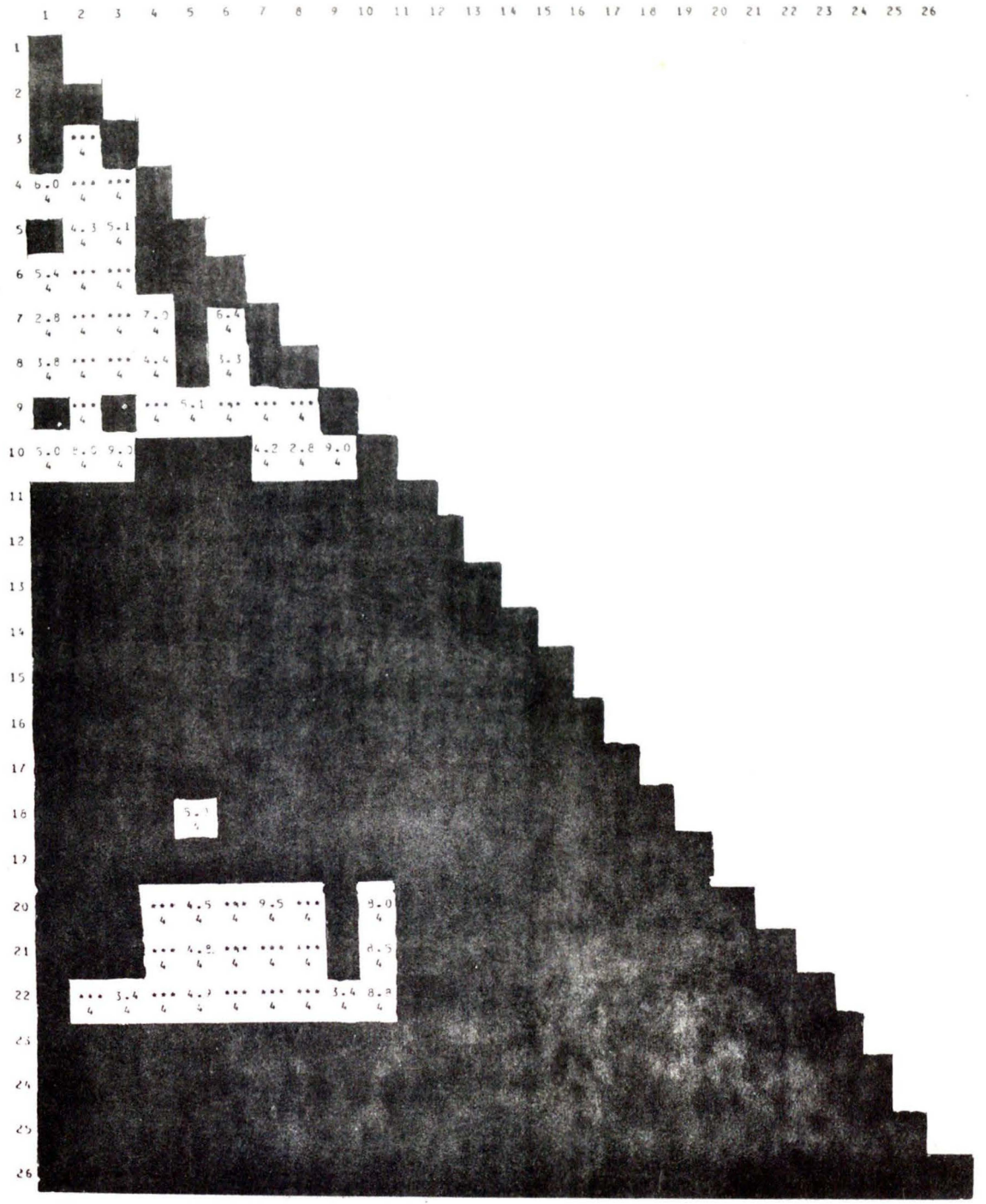

Table 11. T-test statistical comparison of chlorophyll a analyses from Bioassay 1. Treatments with insignificant difference are blocked out. (See Figure 2 for treatment number idenfitication.)

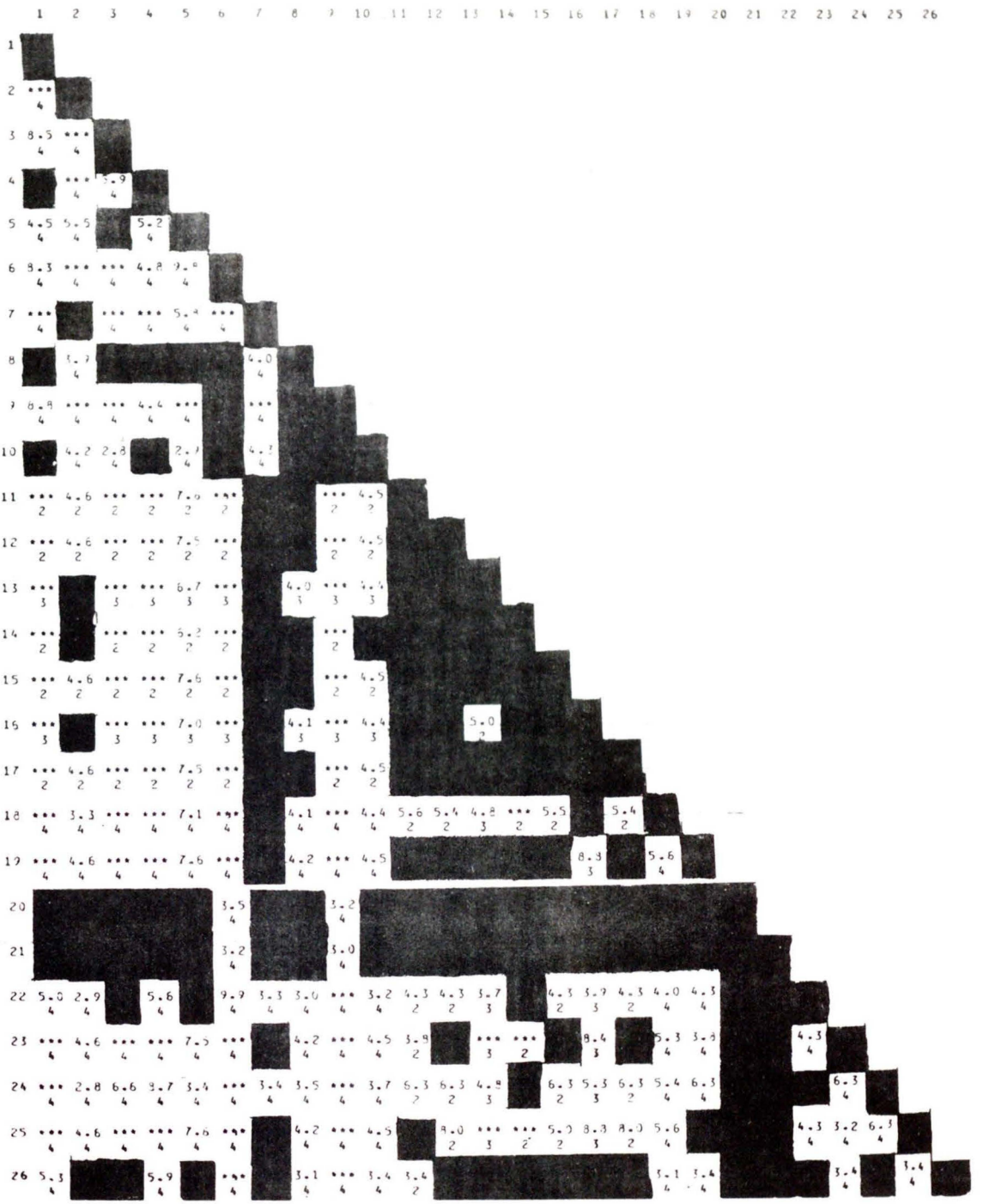

Table 12. T-test statistical comparison of nitrogen fixation analyses from Bioassay 2. Treatments with insignificant difference are blocked out. (See Figure 3 for treatment number identification.)

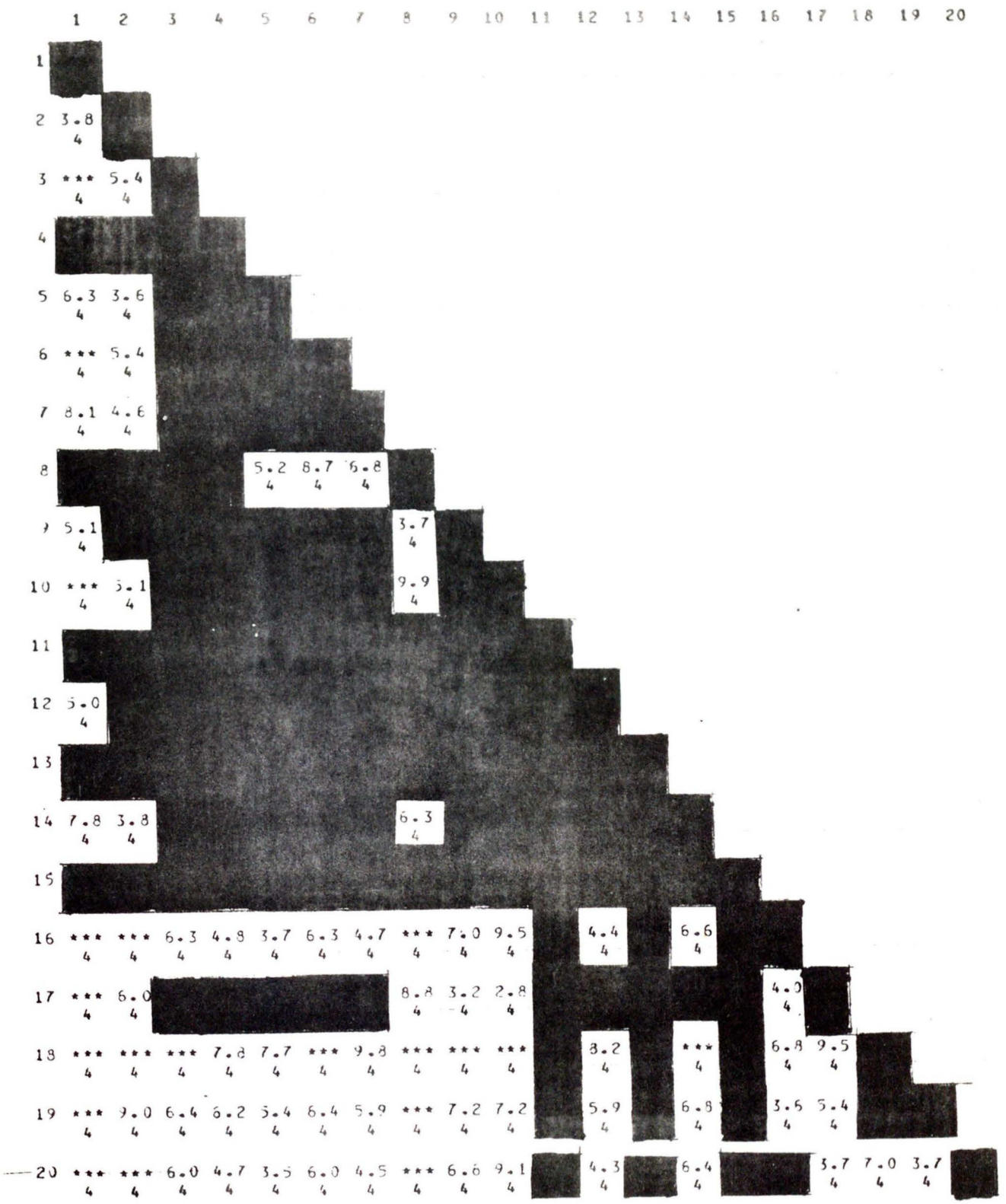
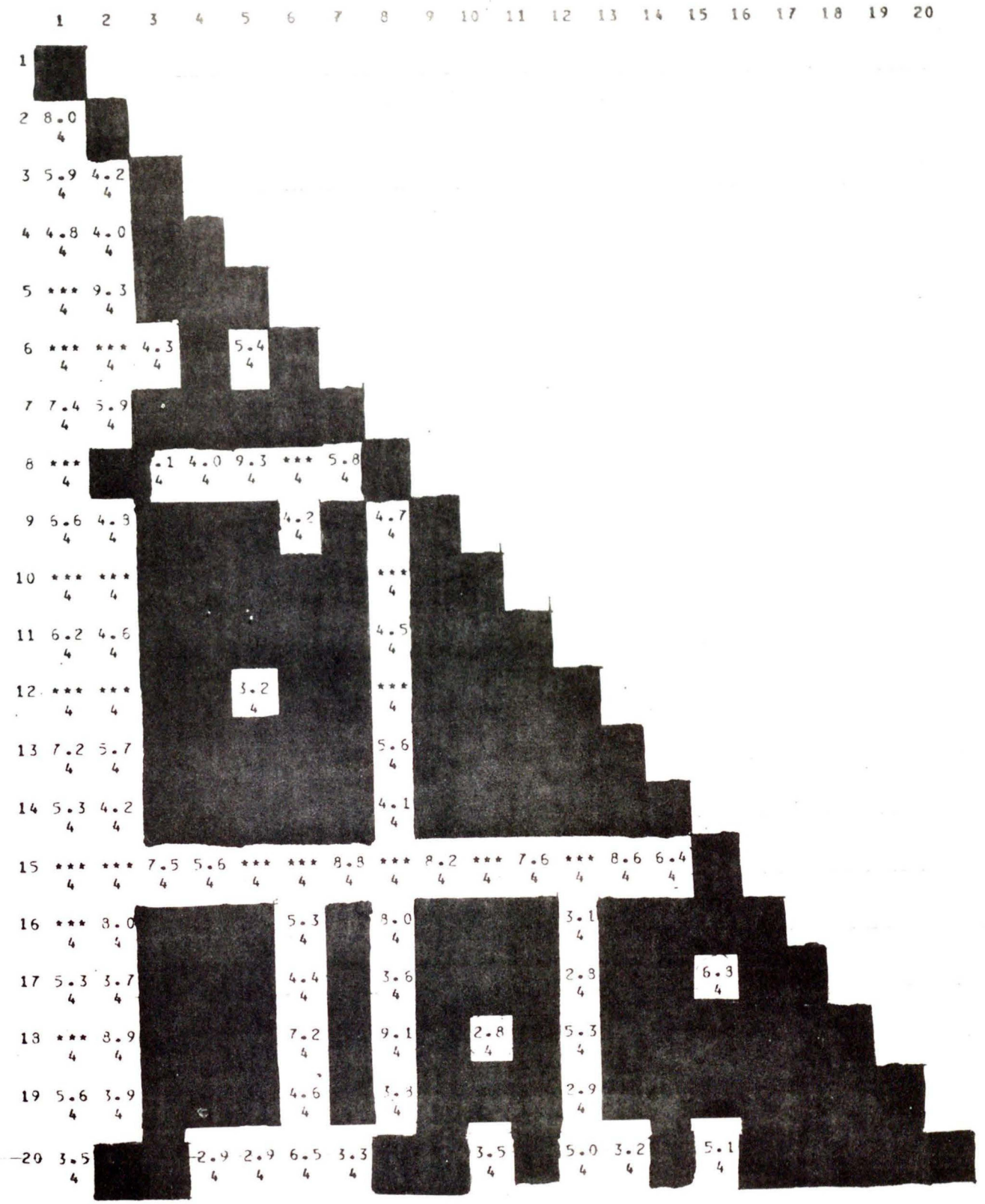
Table 13. T-test statistical comparison of chlorophyll a analyses from Bioassay 2. Treatments with insignificant difference are blocked out. (See Figure 3 for treatment number identification.)